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(54) Title: HUMAN *SYK*

(57) Abstract

The molecular cloning of human *syk* DNA, compositions containing same and uses thereof are disclosed.

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Human *syk*

Background of the Invention

5

Hypersensitivity is the term applied when an adaptive immune response occurs in an inappropriate or exaggerated form causing tissue damage. Specifically, Type I, or immediate hypersensitivity occurs when the immune response is directed against antigens, such as pollen or house dust mite fecal particles. This Type I

10 response is mediated through immunoglobulin E (IgE) interaction with mast cells and basophils. (IMMUNOLOGY, Roitt, I. M., et al., (eds.) C. V. Mosby Co., St. Louis, MO, pp. 19.1-19.20 (1989)).

Mast cells and basophils express the high affinity Fc receptor for IgE, designated polyvalent FceRI. Once IgE binds to the Fcε, subsequent binding of allergen

15 to the IgE molecules caues the receptors to cross-link, triggering the release of cytoplasmic secretory granules containing histamine, and other pharmacological mediators which produce an acute immune reaction (e.g., asthma, dermatitis or rhinitis).

The FcεRI receptor is a complex of three protein subunits, the α, β and γ

20 subunits. The β and γ subunits have an intracellular domain termed a "tyrosine activation motif" or TAM. TAM motifs and related tyrosine-containing motifs are found on a number of growth factor and primary immune response receptors. Phosphorylation of these motifs by protein tyrosine kinases is thought to play an integral role in receptor activation and cellular signal transduction. (Reth, M., Nature

25 338:383-384 (1989); Koch, C. A., et al., Science 252:668-674 (1991); Samuelson, L.E., and Klausner, R.D., J. Biol. Chem. 267:24913-24916 (1992)). It has also been shown that a second group of intracellular tyrosine kinases become physically associated with the activated (phosphorylated) receptors and are likely targets of receptor activity (i.e., phosphorylation by the receptor). Phosphorylation of these "second

30 group" proteins initiates additional intracellular signal transduction events which activate biochemical processes within the cell. (Chan, A.C., et al., Cell 71:649-662 (1992); Chan, A.C., et al., Curr. Opin. Immunol. 4:246-251 (1992); Courtneid, S.A., et al., Cell Growth Differentiation 2:483-486 (1991)).

Thus, the protein-protein interaction of activated receptor protein and

35 intracellular kinase protein plays a critical role in intracellular signal pathways. When these pathways are activated inappropriately, such as when the immune system responds to pollens and other allergens, upper respiratory symptoms of allergic

rhinitis, wheezing and respiratory distress result. In a severe allergic reaction, life-threatening anaphylaxis can occur.

Currently, treatment of allergies includes avoidance of the allergen, such as the removal of pets from the home or the installation of elaborate air filtration systems to

5   minimize airborne allergens. However, complete removal may be difficult such as when the allergen is ubiquitous as in the case of pollen and dust mites. Pharmacological agents, such as anti-histamines and steroids may also be used in treatment. However, these drugs often produce unwanted side effects, such as drowsiness and gastrointestinal distress. When complete removal of the allergen is

10   impossible, and the allergy cannot be managed with drugs, hyposensitization therapy may be tried. Hyposensitization consists of repeated subcutaneous injections of gradually increasing doses of the responsible allergen. However, hyposensitization therapy is a painful and time-consuming process with unpredictable results. (HARRISON'S PRINCIPLES OF INTERNAL MEDICINE, 12th ed., Wilson, J. D., et

15   al., eds., McGraw-Hill, Inc., New York, NY, pp. 1422-1428 (1991)). A significant need exists for materials useful in the design, screening and/or evaluation of effective therapeutic or preventive agents and methods for inhibiting inappropriate or exaggerated response of the immune system to allergens.

20   **Summary of the Invention**

This invention relates to DNA encoding the human form of the protein tyrosine kinase SYK.

In our model, human Syk protein (p72^{syk}, hereinafter "Syk") binds to an IgE receptor protein via one or both of the Syk SH2 domains, and thus plays a key role in

25   a signal transduction pathway resulting in mast cell and basophil degranulation and related events. Mast cell and basophil degranulation leads to the release of histamine and other mediators leading to inappropriate or exaggerated immune responses, such as allergic responses, inflammatory responses and asthma.

Our invention encompasses an isolated DNA molecule encoding part or all of

30   the N-terminal portion of human Syk spanning Met-1 up to Gly-180 of SEQ ID NO:2 and allelic variants thereof. ("Isolated" as the term is used herein means removed or separated from materials with which it is normally associated in nature. An isolated DNA molecule thus comprises a cloned DNA sequence separated from other sequences with which is is normally associated, e.g. in a genome or genetic library.)

35   Such isolated DNA molecules of this invention include molecules comprising part or all of the human *syk* nucleotide sequence spanning A-1 through A-540 of SEQ ID NO:1, or allelic variants thereof.

The isolated DNA molecules of this invention may extend beyond A-540, and include by way of example, DNA molecules comprising the human *syk* nucleotide sequence of SEQ ID NO:1 and allelic variants thereof.

5   A DNA molecule containing the nucleotide sequence of SEQ ID NO:1 or an allelic variant thereof may be cloned as described herein, cloned by other methods using the information disclosed herein, synthesized (e.g. by ligation of overlapping synthetic oligonucleotides), excised from our vector pBS (ATCC Accession No.   ), or combinations of the foregoing.

The DNA sequence depicted in SEQ ID NO:1 was isolated by the mixed 10   oligonucleotide primed amplification of cDNA method (MOPAC). The procedure was carried out in two steps. The first MOPAC step was carried out on a cDNA library obtained from human Daudi cells with degenerate oligonucleotide primers derived from the DNA sequence of the porcine Syk protein described in Taniguchi, T. et al., J. Biol. Chem., 266:15790-15796 (1991). This step resulted in the isolation of a 15   cDNA clone with a cDNA insert containing partial human *syk* cDNA sequence.

The second MOPAC step was carried out on human basophil cDNA using oligonucleotides derived from both the porcine *syk* and the human *syk* partial cDNA sequences. This step also resulted in a partial cDNA sequence. A contiguous human *syk* cDNA sequence was assembled and cloned resulting in a cDNA insert of 1893 20   base pairs (SEQ ID NO:1).

In addition to the sequence of SEQ ID NO:1 we have also identified a number of allelic variants containing the following nucleotide substitutions: T-6, C-15, C-18, C-22, C-1050, G-1287 and G-1323. These and other allelic variants of the nucleotide sequence of our SEQ ID NO:1 are encompassed by this invention.

25   Our invention further includes recombinant DNA constructs consisting of DNA molecules containing the DNA sequence of one of our human *syk* DNA molecules mentioned above and heterologous DNA. "Heterologous" as the term is used herein means DNA (or amino acid) sequence from a different source or origin relative to the sequence in question. Heterologous sequence may thus originate from a 30   non-human source or from human DNA or human peptide sequence not normally or naturally associated with the sequence in question. The heterologous DNA may be directly linked to a human *syk* DNA sequence of this invention to encode a fusion protein or peptide (the terms "protein" and "peptide" are used interchangeably herein) containing human Syk peptide sequence linked via peptide bond to 35   heterologous peptide sequence.

Illustrative vectors and associated methodologies useful for making and using fusion proteins which are suitable for use with the human syk DNA molecules of this

invention include the well known pGEX vectors for GST fusion proteins (see Smith and Johnson, 1988, Gene 67: 31-41), the pRIT2T Protein A Gene Fusion Vector (Nilsson et al, 1985, EMBO J. 4: 1075), the pEZZ 18 Protein A Gene Fusion Vector (Löwenadler et al, 1987, Gene 58:87; Nilsson et al, 1987, Prot Engineering 1:107;

5   Nilsson et al, 1991, Methods Enzymol 198:3) and teh pMC1871 bbeta-galactosidase Fusion Vector (See e.g., Shapiro et al, 1983, Gene 25:71 and Casabadan et al, 1983, Methods Enzymol 100:293).

Recombinant DNA constructs also include, among others, DNA cloning and expression vectors containing a human *syk* DNA molecule of this invention and

10   heterologous DNA. The heterologous DNA may include one or more DNA sequences encoding selectable or amplifiable markers, origins of reliplication, transcriptional control elements (promoter and enhancer sequences) and/or other conventional vector elements. Numerous examples of expression vectors for corresponding host cell types (bacterial, yeast, mammalian, insect, etc.) are well known in the art and have been

15   commercially available for several years. Such vectors containing human *syk* DNA of this invention may be transfected into host cells using known methods. Transfectants may be selected and may be cultured in order to produce the protein encoded by the human *syk* DNA or construct of this invention.

The human Syk protein so obtained (or truncated or fusion proteins containing

20   part or all of the human Syk peptide sequence) may be structurally characterized and so used in the design of compounds capable of inhibiting its signal transducing function. Such materials may also be used in binding assays to identify compounds capable of binding to Syk and thereby interfering with the normal interactions of Syk with other proteins with which Syk can become associated. Human Syk, a potent

25   tyrosine kinase, may also be used as an enzymatic reagent to catalyze the phosphorylation of tyrosine-containing peptides or proteins. In addition, oligonucleotide primers based on the nucleotide sequence of our SEQ ID NO:1 of may be used in diagnostic PCR procedures to identify cells which are expressing Syk or to identify mutations in Syk, e.g. in biopsy or pathology samples.

30   The present invention also relates to the recombinantly produced protein encoded by the human *syk* cDNA sequence. The cDNA insert of the clone described above was sequenced to confirm the open reading frame and the amino acid sequence was deduced. The deduced protein has a sequence of 630 amino acids (SEQ ID NO:2) and a calculated M_r of 72,000. The protein encoded by the human *syk* cDNA

35   sequence contains two src homology region 2 domains (hereinafter SH2 domains) and one tyrosine kinase domain.

The invention also relates to the use of the recombinantly produced Syk protein, or Syk SH2 domain sequences, or fusion proteins, as research reagents to be used in structure-based drug design to develop TAM mimics or other phosphopeptides or other substances which can interfere with the signal transduction

5   cascade of events leading to allergic responses. The Syk protein, or its SH2 domains, can also be used to develop in vitro binding assays to test the TAM mimics, or other peptides, for activity for subsequent use in the methods described herein.

Brief Description of the Drawings

10   Figure 1A and 1B shows the oligonucleotide sequences used in the isolation and cloning of the human syk cDNA.

Figure 2 is a schematic representation of the three-part ligation used to assemble the full-length human syk cDNA.

15   **Detailed Description of the Invention**

Human syk DNA was isolated and cloned as described in detail in Example 1. The mixed oligonucleotide primed amplification of cDNA (MOPAC) PCR method of Lee, C. C., et al. (Science, 1288-1291 (1988)) is the method described herein. However, other standard methods of molecular cloning can also be used.

20   The MOPAC PCR method of cloning the human syk cDNA was performed in two steps. The first step was carried out on a cDNA library obtained from commercially available human Daudi cells (Burkitt's lymphoma cells). Degenerate oligonucleotide primers for the PCR reactions of the first step were derived from the cDNA sequence of the porcine Syk protein described in Taniguchi, T. et al., J. Biol.

25   Chem., 266:15790-15796 (1991). As described in detail in Example 1, this first step resulted in the isolation of a syk cDNA clone with an insert containing a partial sequence of human syk.

When it became apparent that the Daudi cell library did not contain a full length syk cDNA clone, PCR was performed using a human basophil cDNA as a

30   · template in a second series of PCR reactions, also described in detail in Example 1. A contiguous human syk cDNA was assembled via a three-part ligation and transformed into competent bacteria, as described in Example 1 and depicted in Figure 2. A plasmid, pBS SK(-) hu syk, was subjected to DNA sequencing analysis to confirm that the correct open reading frame of 630 codons for human Syk was present. The

35   nucleotide sequence of the cDNA, 1893 base pairs, is identified in SEQ ID NO:1. The deduced amino acid sequence of human Syk is also shown in SEQ ID NO:2. The sequence has 630 amino acids with an M_r of approximately 72,000.

The deduced human Syk protein sequence exhibits homology with the porcine Syk protein sequence described in Taniguchi, T. et al., J. Biol. Chem., 266:15790-15796 (1991). Neither deduced amino acid sequence contains a ligand binding or membrane-spanning region, suggesting that the Syk protein is not associated with the cell

5   membrane (i.e., a non-receptor protein). However, both human and porcine Syk contain two src homology region 2 domains (SH2 domains), one at the N-terminal ($SH2_N$) and one at the C-terminal ($SH2_C$) and one protein tyrosine kinase domain.

Src homology region 2 (SH2) domains are noncatalytic domains that are conserved among a series of cytoplasmic signaling proteins regulated by receptor

10   protein tyrosine kinases (Koch, C.A., et al. Science 252:668-674 (1991). The SH2 domain contains approximately 100 amino acids and was first shown to be a nonkinase domain conserved between the src and fps gene products. (Songyang, Z., et al., Cell 72:767-778 (1993). Now, more than 20 cytosolic proteins likely to be involved in signaling events have been shown to contain SH2 domains. (Songyang, Z.,

15   et al., Cell 72:767-778 (1993). The SH2 domains of these signaling proteins bind tyrosine phosphorylated polypeptides in a sequence specific manner.

Tyrosine phosphorylation of a receptor, (i.e., activation of a receptor) acts as a switch to induce the binding of these signaling protein kinases via their SH2 domains, thereby mediating the formation of heteromeric protein complexes at, or near, the

20   plasma membrane. The formation of these complexes is thought to control the activation of intracellular signal transduction pathways. (Koch, C.A., et al. Science 252:668-674 (1991).

A number of primary immune response receptors on T cells and B cells, and the IgE receptor on mast cells and basophils share a common amino acid motif present

25   in their cytoplasmic tail. (Samuelson, L.E. and Klausner, R.D., J. Biol. Chem. 267:24913-24916 (1992); Reth, M., Nature 338:383-384 (1989)). This common motif, 18-27 amino acids long, has been termed the "tyrosine activation motif" or TAM.

The function of the TAM motif has been studied for the T cell receptor (TCR). (Romeo, C. et al., Cell 68:889-897 (1992)). Activation of the TCR by antigen results in

30   crosslinking of TCR ζ subunits, leading to phosphorylation of a TAM in the TCR by a protein kinase. The phosphorylation of TAM motifs is believed to be critical for TCR activation because of evidence that the substitution of a phenylalanine residue for either tyrosine residue in the TAM motif of the ζ subunit of the TCR blocked T cell activation of IL-2 production. (Letourner, F. and Klausner, R.D., Science 255:79-82

35   (1992)).

After phosphorylation, the activated TCR has been shown to be associated with a 70 kD protein tyrosine kinase, termed ZAP-70, which also undergoes

subsequent phosphorylation. (Chan, A. C., et al., Cell 71:649-662 (1992). It is believed that assembly of the ZAP-70 protein-TCR complex, via the ZAP-70 SH2 domains interaction with phosphorylated TCR TAM motifs, results in additional levels of regulation of TCR signaling. (Chan, A. C., et al., Cell 71:649-662 (1992).

5   It has also been shown that activation of rat basophil leukemia cells (RBL cells) by antigen cross-linking of IgE molecules bound to the IgE receptor (FcεRI) results in rapid and sustained activation of tyrosine phosphorylation of multiple RBL proteins. (Benhamou, M.V., et al., J. Biol. Chem. 267:7310-7314 (1992); Li, W., et al., Mol. Cell Biol. 12:3176-3182 (1992)). Two protein tyrosine kinases have been

10   implicated in these phosphorylation events. The first kinase, lyn, is a protein tyrosine kinase belonging to the family of src protein kinases. (Wiseman, E., and Bolen, J.B., Nature 355:78-80 (1992); Seidel-Dugan, C., et al., Mol. Cell Biol. 12:1835-1845 (1992)). Lyn is activated (phosphorylated) transiently (1-5 min) following antigen incubation and appears to co-precipitate with the IgE receptor in immunoprecipitation

15   experiments.

A second protein tyrosine kinase, Syk, has been shown to be activated after IgE receptor crosslinking with antigen and is associated in a complex with the IgE receptor (Hutchcroft, J.E., et al., Proc. Natl. Acad. Sci. USA 89:9107-9111 (1992)). In our model, Syk binding to the IgE receptor is mediated by binding to phosphorylated

20   TAM motifs in the β or γ subunit of the IgE receptor. Assembly of this human Syk protein-IgE receptor complex results in regulation of IgE receptor signaling, such as mast cell degranulation.

In our model, lyn phosphorylates the tyrosine residues of the TAM motifs of the β and γ subunits of the IgE receptor. Syk then binds to the phosphorylated TAM

25   motifs through its SH2 domains. Activation (i.e., phosphorylation) of Syk can be mediated by this binding, by lyn phosphorylation, or another mechanism. In any event, the assembly of a Syk protein-IgE receptor complex, via the syk SH2 domains interaction with the IgE receptor TAM, results in additional levels of regulation of IgE receptor signaling.

30   Inhibition of the interaction of Syk and the IgE receptor, e.g. via the Syk SH2 domains and the receptor phosphorylated TAM motif, interrupts the cascade of signaling events leading to mast cell or basophil degranulation, allergic responses, inflammatory responses or any other inappropriate or exaggerated immune response.

For example, molecules that specifically interfere with the interaction of the

35   syk SH2 domain and the TAM motif would mimic the structure of the TAM binding site (peptide mimics), and bind to the SH2 domain of Syk, thereby preventing Syk

from binding to TAM on the receptor and, thus, preventing, reducing, or completely eliminating the response of the receptor to the stimuli.

TAM motifs from different receptor subunits appear to specifically activate distinct biological responses in different cell types. Therefore, TAM mimics are

5   designed to mimic the TAM motifs of specific receptors. For example, the TAM motif of the ζ subunit on the T cell receptor is different from the TAM motif on the β and γ subunits on the IgE receptor of mast cells. Thus, a TAM mimic for the TCR TAM would not be as effective in inhibiting the binding of Syk to the IgE receptor.

In one embodiment, the activation of mast cells and basophils leading to

10 degranulation, is inhibited with a peptide which mimics a phosphorylated TAM of the β or γ subunit of the IgE receptor. Such TAM mimics can be synthesized according to the methods disclosed in U.S.S.N. 08/013,414, the teachings of which are incorporated herein by reference.

The TAM mimics can inhibit the activation of mast cells and basophils by

15 blocking the association of the Syk protein with the activated IgE receptor. They mimic the phosphorylated TAM on the IgE receptor and bind directly to the SH2 domain of the Syk protein. Thus, the TAM mimics act as competitive antagonists.

The TAM mimics used in the methods described herein can be peptides (comprised of natural and non-natural amino acids) or can be peptide analogs

20 (comprised of peptide and non-peptide portions). All TAM mimics used in these methods have specific characteristics pertaining to biological activity. These characteristics include the ability of the phosphotyrosine residues, to bind to the SH2 domain of Syk, and retention of a biologically active conformation.

The TAM mimics used in the methods described herein include at least five

25 amino acid residues and generally have a sequence in the range of 15-39 amino acid residues. However, longer mimics can be used (e.g., up to the total length of an SH2 domain which is characteristically approximately 100 amino acid residues) if they have the desired characteristics described above. Shorter mimic sequences would have the distinct advantage that they exhibit lesser charge than longer sequences which

30 would facilitate membrane penetration and cell entry. Shorter mimics would also be less susceptible to enzymatic degradation and less subject to the conformational constraints required for biological activity.

Although the methods described herein preferably use TAM mimics, peptide and peptide analog mimics of the SH2 domains of the Syk protein can also be used in

35 the methods described herein. The SH2 mimic would also block the association of the Syk protein with the activated IgE receptor as a competitive antagonist, thus,

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inhibiting mast cell degranulation or other inappropriate or exaggerated immune responses.

TAM mimics and SH2 mimics can be screened for biological activity (i.e., the ability to block the association of syk with the activated receptor) using art-

5   recognized in vitro assays. For example, peptide capture assays can be used in which recombinant SH2 domains (for TAM mimic screening) or TAM sequences (for SH2 screening) are immobilized in plastic microtiter wells. Labeled non-phosphorylated or phosphorylated peptides are then added to these wells, and incubated under conditions sufficient for binding to take place. Unbound labeled peptide is then

10   removed and the amount of bound peptide is determined. The TAM or SH2 mimics exhibiting the highest per cent binding to bound peptide have the greatest potential for use in the methods described herein.

Biospecific Interaction Analysis (BIA) in real time can also be performed to evaluate biological activity. Surface plasmon resonance (SPR), which is the basis for

15   BIA measurements, is an optical phenomenon arising in metal films under conditions of total internal reflection. The phenomenon produces a sharp dip in the intensity of reflected light at a specific angle. The position of this resonance angle depends on several factors, including the refractive index of the medium close to the non-illuminated side of the metal film. Refractive index is directly related to the

20   concentration of dissolved material in the medium. By keeping other factors constant, SPR is used to measure changes in the concentration of macromolecules in a surface layer of solution in contact with a dextran-coated gold film. As described in detail in Example 2, using the BIAcore^{TM} instrument from Pharmacia Biosensor AB, the association and dissociation rate constants for a peptide binding to the SH2 domain

25   of a protein kinase, PI3K, have been measured. The association and dissociation rate constants for peptide mimics binding to SH2 domains of Syk can also be measured under similar conditions. Peptide mimics exhibiting higher association constants (K_a) have the greatest potential for use in the methods described herein.

In particular, mast cell or basophil degranulation assays can be used to

30   measure in vitro activity of TAM or SH2 mimics. The release of histamine, the release of beta-hexosaminidase, the release of cytokines and/or increased phosphatidylinositol hydrolysis or tyrosine phosphorylation can be detected in in vitro assays as an indication of biological activity. (Stephan, V.M., et al., J. Biol. Chem. 267:5434-5441 (1992)). For example, histamine release can be measured by

35   radioimmunoassay using a kit available from AMAC Inc. (Westbrook, ME).

A known mast cell/basophil degranulation assay is described in detail in Example 3. Briefly, mast cells and basophils express the FcεRI receptor on their cell

surface. This receptor binds the Fc portion of IgE antibody with high affinity. Binding of multivalent antigens activates the process that leads to degranulation (the release of preformed secretory granules) and the stimulation of the production of a number of cytokines. The assay has two variations: an intact cell assay and a permeabilized cell

5   assay. For testing peptides and small molecules where membrane permeability is questionable, the cells are permeabilized using streptolysin O. Tyrosine kinase inhibitors, such as leflunomide and its active metabolite, A771726, vanadate and genistein, are used as controls to establish whether known inhibitors of mast cell degranulation show inhibitory activity. Thus, if a TAM mimic interferes with

10   degranulation, it has the greatest potential for use in the methods described herein.

TAM mimics can also be evaluated for biological activity using immunoprecipitation assays or receptor association assays such as described in Matsuda, M., et al., J. Biol. Chem. 268:4441-4446 (1993) and Escobedo, J. A., et al., Mol. Cell. Biol. 11:1125-1132 (1991). The basic protocol is to lyse the cells of interest

15   in a gentle buffer with detergent that would preserve natural in vivo protein-protein interactions, creating a detergent solubilized cytoplasmic lysate, and a particulate/detergent insoluble fraction. The detergent soluble lysate is then added to a quantity of human Syk SH2 domain fusion protein and mixed gently for a period of time sufficient to allow relatively high affinity interactions to form between the introduced

20   fusion protein and any cellular proteins. The fusion proteins are purified by centrifugation from the lysate, and washed several times with lysate buffer to remove any non-specific or low affinity interactions. The proteins that remain associated with the fusion protein are then dissociated from the fusion protein, and analyzed by denaturing gel electrophoresis. The eluted proteins are then visualized by any number

25   of methods. For example, a western blot of the SDS gel, probed with anti-phosphotyrosine antibody, can be used to visualize phosphotyrosine containing proteins.

The cells can also be metabolically labeled (that is, "fed" label in culture) so as to label either all proteins (with ^{35}S labeled methionine and cysteine) or to label

30   phosphoproteins (with ^{32}P orthophosphate). After the denaturing gel electrophoresis, the proteins are visualized by autoradiography.

Antibodies specific for various components of the mast cell IgE receptor complex can be used to directly determine, either by immunoprecipitation or western blot, whether those specific components are interacting with the SH2 fusion proteins

35   in extracts of either resting or activated RBL cells by addition of IgE and hapten. Differences in the proteins associated with the SH2 fusion proteins should be observed between resting and activated cells, reflecting the changing interactions of

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human Syk upon mast cell activation. By identifying those proteins that associate, or disassociate, upon activation, one may identify cellular proteins that directly interact with human Syk during mast cell activation.

5   Moreover, antibodies, either polyclonal or monoclonal, can be produced that are immunoreactive with one or both of the SH2 domains of the Syk protein. Such antibodies can be made by standard laboratory techniques. By screening the SH2 antibodies, one may obtain specific antibodies which also block the association of the Syk protein with the activated IgE receptor thus, inhibiting mast cell degranulation or other inappropriate or exaggerated immune responses.

10   Anti-Syk SH2 domain antibodies can be evaluated for biological activity using standard laboratory techniques such as radioimmunoassay or ELISA. Immunoprecipitation assays, or receptor association assays, such as those described above, can also be used.

TAM mimics, SH2 mimics, and antibodies demonstrating the desired activity

15   in vitro can be tested in vivo for inhibitory activity. For example, TAM mimics can be tested in suitable animal model systems including rats, mice, or horses. There are numerous animal models of asthma that have been developed and can be used (for reviews, see Larson, "Experimental Models of Reversible Airway Obstruction", in THE LUNG, Scientific Foundations, Crystal, West et al. (eds.), Raven Press, New York, pp.

20   953-965 (1991); Warner et al., 1990, Am. Rev. Respir. Dis. 141:253-257). Species used as animal models for asthma include mice, rats, guinea pigs, rabbits, ponies, dogs, sheep and primates. Other in vivo models available are described in Cross et al., Lab Invest. 63:162-170 (1990)); and Koh, et al., Science, 256:1210-1213 (1992)).

TAM mimics, SH2 mimics and antibodies which inhibit degranulation of mast

25   cells and basophils are preferred for treatment or prevention of type I allergic (IgE-mediated) reactions such as asthma and allergic rhinitis, diseases and disorders associated with an inappropriate or exaggerated immune system response, or inflammation. The preferred method of treatment, or prevention, is by administrating to a mammal an effective amount of a peptide, or peptide analog which mimics a

30   phosphorylated TAM which blocks the association of the Syk SH2 domain with the activated IgE receptor.

Diseases and disorders which can be treated by administration of an effective amount of a TAM mimic, SH2 mimic, or antibody which inhibits inappropriate or exaggerated inflammatory/immune responses include inflammatory arthritis (e.g.,

35   rheumatoid arthritis, seronegative spondyloarthritites (Behcets disease, Reiter's syndrome, etc.), juvenile rheumatoid arthritis, vasculitis, psoriatic arthritis, polydermatomyositis); systemic lupus erythematosus (SLE); asthma; inflammatory

dermatoses (e.g., psoriasis, dermatitis herpetiformis, eczema, necrotizing and cutaneous vasculitis, bullous diseases); inflammatory bowel disease (e.g., Crohn's disease and ulcerative colitis); tissue damage relating to tissue transplantation; and other autoimmune disorders.

5   Additional disorders such as glomerulonephritis, juvenile onset diabetes, multiple sclerosis, allergic conditions, autoimmune thyroiditis, allograft rejection (e.g., rejection of transplanted organs such as kidney, heart, pancreas, bowel or liver), and graft-versus-host disease can also be treated.

Furthermore, in a preferred aspect of the invention, a TAM mimic which

10   inhibits mast cell and basophil degranulation is administered to treat, or prevent, a type I allergic reaction such as one or more of the following: atopic diseases (e.g., allergic rhinitis, commonly known as hayfever, due to pollens, fungal spores, dust, animal dander, asthma, atopic dermatitis, and allergic gastroenteropathy due to ingested food); anaphylaxis (a systemic immediate hypersensitivity affecting multiple

15   organs, due to, for example, drugs, proteins such as in vaccines, and nonproteins such as antibiotics, anesthetics, salicylates, foods, venom of stinging insects); and urticaria or a angioedema (increased cutaneous vascular permeability). Mammals include horses, cattle, dogs, cats and humans.

Various delivery systems are known and can be used to administer a TAM

20   mimic. For example, encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis, construction of a TAM mimic-encoding nucleic acid as part of a retroviral or other vector can be used. Methods of introduction for both peptide mimics and antibodies include, for example, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal,

25   epidural, and oral routes. Any other convenient route of administration can be used, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and the TAM mimic may be administered together with other biologically active agents. Administration can be systemic or local.

30   For TAM mimics which are used for inhibition of mast cell activation, e.g., for therapy of asthma or allergy, the preferred route of administration is nasal or via a bronchial aerosol. In particular, a bronchial aerosol is employed. Pulmonary administration can be accomplished, for example, using any of various delivery devices known in the art (Newman, S.P., AEROSOLS AND THE LUNG, Clarke and

35   Davia (eds., Butterworths, London, England, pp. 197-224 (1984); PCT Publication No. WO 92/16192, PCT Publication No. WO 91/08760. Delivery devices include, for example, nebulizers, metered dose inhalers, and powder inhalers. Various delivery

devices are commercially available and can be employed, e.g., Ultravent nebulizer (Mallinckrodt, Inc., St. Louis, Missouri); Acorn II nebulizer (Marquest Medical Products, Englewood, Colorado), Ventolin metered dose inhaler (Glaxo Inc., Research Triangle park, North Carolina); Spinhaler powder inhaler (Fisons Corp., Bedford,

5   Massachusetts). Such devices typically entail the use of formulations suitable for dispensing from such a device, in which a propellant material may be present.

A nebulizer may be used to produce aerosol particles, or any of various physiologically acceptable inert gases may be used as an aerosolizing agent. Other components such as physiologically acceptable surfacatants (e.g., glycerides),

10   excipients (e.g., lactose), carriers, and diluents may also be included.

In another embodiment, it may be desirable to administer the peptide mimics or antibodies of the invention locally to the area in need of treatment. This may be achieved by, for example, local infusion during surgery, topical application (e.g., for skin conditions such as psoriasis), by injection, by means of a catheter, by means of a

15   suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

The invention also relates to the purified recombinantly produced intact Syk protein and purified recombinantly produced SH2 domains. Expression vectors can be constructed using routine laboratory methods to express the intact human Syk

20   protein, or any of its various peptide domains, especially the SH2 domains. Proteins and peptides can be expressed as a component of a fusion protein, such as a protein in which the other component is a "tag" such as with a glutathione-S-transferase (GST) enzyme tag or a polyhistidine tag, to facilitate purification. Alternatively, the proteins can be expressed alone, (i.e., not as a member of a fusion protein). Proteins expressed

25   as fusion proteins or alone, can be purified by standard laboratory methods, such as column chromatography or electrophoretic techniques.

A fusion protein in which the C-terminal SH2 domain of human Syk is linked to GST has been produced as described in Example 4. In this embodiment, the C-terminal SH2 domain was cloned into the pGex fusion vector system. This vector

30   provides a fusion site with GST at the N-terminus, followed by a protease cleavage site directly preceding the protein to be fused. The fusion proteins are generated in E. coli, and purified by absorption to agarose beads containing glutathione.

The SH2 domain constructs can be assayed for their activity using the in vitro transcription/translation assay as described in Example 5. The purpose of this assay

35   is to determine if the SH2 domains are functional via binding to phosphotyrosine and to develop additional assays to screen and identify peptides and peptide mimics which bind to these SH2 domains.

The purified recombinant Syk protein and Syk SH2 domains can be used to determine the affinity of the two Syk SH2 domains for binding to the TAM motifs from the β and γ subunits of the IgE receptor and to determine the minimal region of the TAM motif that can bind to the Syk SH2 domain. This information can be used

5   for the design of inhibitors that block IgE receptor activation.

The recombinant Syk protein and Syk SH2 domains can also be used to determine the structure of the phosphorylated TAM motif when bound to the Syk SH2 domains. For example, in order to design small molecules that mimic the structure of the phosphorylated TAM motif, it is helpful to know the structure of the TAM

10   peptide sequence when bound to the Syk SH2 domain. Since short peptide sequences like the TAM motif have flexible structures in solution, it is useful to examine their structure when bound to their binding partner in order to design molecules that mimic their structure. The structure of the phosphorylated TAM motif bound to Syk SH2 domains can be derived using X-ray crystallography and NMR spectroscopy. This

15   structural information can be used to design additional TAM mimics. Once these mimics are synthesized, their structure can be examined when bound to Syk SH2 domains in order to further optimize the design of molecules that "best fit" in Syk.

The recombinant Syk protein and Syk SH2 domains can further be used to derive assays that determine the binding affinity of the TAM mimetic compounds for

20   Syk SH2 domains. These assays can be used to determine whether candidate inhibitory molecules interfere with the binding of Syk SH2 domains to phosphoTAM motifs from the β and γ TAM motifs.

Moreover, variant recombinant sequences of the Syk protein and SH2 domains can be constructed for use in dominant negative expression studies in which mutant

25   forms of the Syk protein, or Syk SH2 domains, are expressed that dominantly interfere with the binding of wild type Syk protein with the activated IgE receptor. Studies such as these can lead to further elucidation of the signal transduction cascade of events.

The present invention will now be illustrated by the following examples which

30   further and more specifically illustrate the invention.

Example 1: Isolation and Cloning of a Human syk

A λ gt10 cDNA library derived from Human Daudi cells' (Burkitt's Lymphoma, ATCC# CCL 213) mRNA was purchased from a commercial source

35   (Clontech Laboratories Inc., Catalog #HL 1117a, random + oligo dT primed). Phage from the bacterial lysate were used as a template to amplify the library in the MOPAC PCR reaction described by Lee, C.C. et al., Science 239:1288-1291 (1988).

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Primers for the aforementioned PCR reactions were derived from the DNA sequence
of the porcine pp72 *syk* protein described by Taniguchi, T. *et al.*, J. Biol. Chem.
266:15790-15796 (1991).

5   Two pairs of degenerate primers were synthesized based on relatively non-
degenerate regions of the porcine *syk* cDNA: 1) the 5' end of the NH2 terminal SH2
domain (sense primer) and the 5' end of the COOH terminal SH2 domain (antisense
primer) and 2) a portion of the kinase domain spanning about 200 amino acids. These
primers labeled S1, AS2 and S3, AS4 respectively, (SEQ ID NOS: 3-11), are depicted
in Figure 1A and 1B. To reduce the degeneracy of these oligo primer pairs, multiple
10   versions of each oligo were synthesized and used in multiple combinations in MOPAC
PCR so that at least the last five 3' bases would exactly match the *syk* cDNA in at
least one reaction. Hence the oligonucleotide primers listed in Figure 1A and 1B are
S1A, S1B, S1C, (SEQ ID NOS: 3, 4, 5) and AS2A, AS2B (SEQ ID NOS: 6, 7), etc.

         All reactions were performed in duplicate in an MJ Research Minicycler using
15   the following cycling program at two different annealing temperatures (55°C or 60°C)
under these ionic conditions:

50 mM KCl	95°C 5 min., add 2.5 units Taq. pol.	
10 mM Tris-HCl pH 8.3 /	93°C 40 seconds	-denaturation
1.5 mM MgCl$_2$ 40x	55°C or 60°C 40 secs.	-annealing
0.001 (wt./vol.) gelatin \	75°C 40 seconds	-polymerization
200 µM each dNTP	75°C 2 minutes	
1 µM sense primer	4°C hold indefinitely	
1 µM antisense primer		
1 µl λ phage library		
100 µl total reaction volume		

10 µl aliquots of each reaction were analyzed by agarose gel electrophoresis for
amplification of DNA fragments of the predicted size. No amplifications products
30   were observed in reactions using the any of the S1 (SEQ ID NOS: 3-5) and AS2 (SEQ
ID NOS: 6 and 7) primer pairs based on the SH2 region of Syk. However, DNA
fragments approximately 600 base pairs in length, sufficient to encode approximately
200 amino acids of the kinase domain of Syk, resulted from amplifications employing
the S3B (SEQ ID NOS: 8 and 9) and AS4A (SEQ ID NOS: 10 and 11) primer pairs.

35   The remaining 90 µl of the S3B/AS4A reaction were phenol-chloroform
extracted, ethanol precipitated and digested with the restriction endonucleases Xho I
and Cla I which had been included at the 5' ends of the sense and antisense

oligonucleotide primers, respectively. Simultaneously, the vector Bluescript KS+ was digested with Xho I and Cla I. DNA fragments from both reactions were resolved on a 0.5% (wt./vol.) low-melt agarose (NuSieve GTG or SeaPlaque GTG, FMC Corp.) gel, excised, melted at 70°C, and ligated for one hour at room temperature with T4 DNA

5   ligase (Pharmacia). The ligations were melted at 70°C and transformed into competent DH5a bacteria (Gibco-BRL) according to the manufacturer's instructions, the bacteria spread onto BHI (Difco) agar plates supplemented with 200 µg/ml ampicillin (Sigma Corp.), and the plates incubated overnight at 37°C. Six colonies were inoculated into 4 ml BHI cultures supplemented with 200 µg/ml ampicillin, and

10   the 4 ml cultures were shaken overnight at 37°C. Plasmid DNA was prepared using a mini-prep kit (Qiagen Corp.), subjected to digestion with Xho I and Cla I, and analyzed by agarose gel electrophoresis. Plasmid from three of the colonies were positive for an Xho I/Cla I fragment roughly 620 bp in length, and were subsequently subjected to double stranded DNA dideoxy sequence analysis (Sequenase 2.0, U.S.

15   Biochemicals). The DNA sequence of the 5' ends of the inserts within the three plasmids was identical. Translation of the DNA sequence revealed extensive homology with the porcine Syk protein sequence. Within the first 47 amino acids of the human *syk* partial cDNA clones, the only difference detected with porcine *syk* was position 481 with a substitution of serine for cysteine.

20   In an attempt to clone the 5' end of the human *syk* cDNA, nested antisense nondegenerate oligonucleotide primers derived from the 5' end of the aforementioned human *syk* partial cDNA clone were synthesized (ASA (SEQ ID NO: 13) and ASB (SEQ ID NO: 14), Figure 1A and 1B). These were to be used in conjunction with λ left or λ right arm primers (from the region bounding the Eco RI cloning site in λ gt10) to

25   amplify the 5' end of the insert in phage harboring human *syk* cDNA from the Daudi λ gt10 library. To verify the authenticity of PCR products as *syk* cDNAs, another oligonucleotide, probe Z (SEQ ID NO: 12), was synthesized for use in Southern and colony hybridization analysis.

            Primary PCR reactions were carried out using either the λ for 1°/ASB (SEQ ID

30   NOS: 15 and 14 respectively) or λ rev 1°/ASB (SEQ ID NOS: 17 and 14 respectively) primer pair with the program described above, with the exception that the annealing temperature was either 60°C or 65°C and polymerization was allowed to proceed for 2 minutes 30 seconds. A secondary reaction ensued, using 1 µl of the primary reaction as template, with either the λ for 2°/ASA (SEQ ID NOS: 16 and 13 respectively) or λ

35   rev 2°/ASA (SEQ ID NOS: 18 and 13 respectively) primer pair, and the same annealing temperature used in the primary amplification. Agarose gel electrophoretic analysis of the secondary PCR products revealed a smear from approximately 2 kb to

the bottom of the gel, with discrete bands scattered throughout the smear in the λ rev
2°/ASB reaction. An alkali transfer of the gel was made onto charged nylon
membrane (Qiabrane Plus, Qiagen Inc.) using a BioRad 785 Vacuum Blotter according
to the manufacturer's protocol. After neutralization the blot was placed in

5   oligohybmix (6 X SSC, 5 X Denhardt's solution, 1% SDS, 10 mM $NaPO_4$, 200 µg/ml
denatured salmon sperm DNA) and allowed to prehybridize for one hour at 50°C. Z
probe was labeled with ^{33}P-γ-ATP using T4 polynucleotide kinase (Pharmacia) under
standard conditions (Sambrook, et al., Molecular Cloning: A Laboratory Manual
(Cold Spring Harbor Lab., Cold Spring Harbor, NY) (1989)). The probe was purified

10   from unincorporated ^{33}P using a spin column made with Sephadex G-25 (Pharmacia),
and added to the blot. Hybridization at 50°C proceeded overnight, after which the
blot was washed 3 X 20 minutes at 55°C in 2 X SSC/1% SDS and subjected to
autoradiography. Probe hybridized to the blot in a pattern that mirrored the smear
with discrete bands visualized by ethidium bromide staining of the gel, indicating that

15   the amplified DNA products were derived from human syk cDNA.

       The remainder of the λ rev 2°/ASA (65°C) secondary reaction was
phenolchloroform extracted, ethanol precipitated and digested with the restriction
endonucleases Xho I and Cla I and resolved on a 0.5% (wt./vol) low-melt agarose gel.
A band of approximately 1.2 kb was excised, melted at 70°C, and ligated into Xho

20   I/Cla I digested Bluescript KS+ plasmid. Plasmid prepared from colonies arising
from transformation of the ligation was analyzed by agarose gel electrophoresis after
digestion with Xho I and Cla I restriction endonucleases. Several of the plasmids
contained 1.2 kb inserts whose 5' ends were analyzed by DNA sequence analysis.
The amino acid sequence deduced from these partial cDNAs is quite homologous with

25   that of porcine Syk, and extend NH_2 terminal to the serine 79 residue of the protein.
Hence the amplified partial cDNA was roughly 240 bp too short to encode the 5' end
of the human syk cDNA.

       Further attempts made to isolate a partial cDNA with the 5' end of the human
syk gene were performed in an analogous manner. Nested antisense nondegenerate

30   oligonucleotide primers derived from the 5' end of the above partial cDNA clone were
synthesized (ASX and ASY (SEQ ID NOS: 20 and 21), Figure 1A and 1B) in
conjunction with λ left or λ right arm primers. DNA fragments generated using these
primer pairs hybridized to Probe W (SEQ ID NO: 19) on Southern blots, proving the
fragments were specific for the 5' end of human Syk. However, none of the amplified

35   fragments were greater than 180 bp in length, and were therefore insufficient to encode
the initiator codon of human Syk.

Concomitantly, 800,000 plaques of the λ gt10 Daudi cell cDNA library were
screened with ^{32}P nick-translated (Stratagene Prime-It) 0.6 kb fragment from the
human *syk* kinase domain (S3B/AS4A). Hybridization and washing of duplicate
filters (Hybond N+, Amersham) according to Church and Gilbert protocol buffers, at

5   65°C led to 55 initial positives, 27 of which were positive on secondary plating and
hybridization as above.

Eleven of the secondaries were screened for insert size by PCR amplification,
using the *syk* kinase domain oligo ASB with the λ for 1° or λ rev 1° primers. Four
clones yielding the largest PCR products were chosen for further characterization by

10   subcloning and sequencing. (D1.2, 3.2, 8.2, 10.2) From these λ clones, partial sequence
of human *syk* cDNA, from the COOH-terminal SH2 domain to the 3' untranslated
region, was determined.

When it became apparent that the Daudi cell λ gt10 library probably did not
contain a full length *syk* cDNA clone, PCR was performed using human basophil

15   cDNA as template in order to obtain the gene's 5' end. Basophils were purified from
60 ml of freshly drawn blood using Percoll step gradient according to the protocol of
Warner, J.A., et al. J. Immunol. Methods 105:107-110 (1987). Basophils removed from
the gradient's interface were washed 2 X in PBS + 4 mM EDTA, and the cell pellet
lysed in guanidine thiocyanate buffer (Chirgwin, J.M., et al. Biochem. 18:5294-5299

20   (1979)). RNA was purified by acid phenol extraction. One tenth of the total RNA
was reverse transcribed with Superscript reverse transcriptase (Gibco-BRL) according
to the manufacturer's instructions.

A pair of "guessmer" oligonucleotides based on the first nine amino acids of
porcine Syk with biased codon selection (Syk ATG1 and Syk ATG2, (SEQ ID NOS: 22

25   and 23), Figure 1A and 1B) were synthesized for use as a primers in conjunction with
the antisense primers ASX and ASY (SEQ ID NOS: 20 and 21) in PCR amplification
of the 5' end of human *syk*. The design of these guessmers was aided by the
consideration of nucleotide sequence of the first nine amino acids of the Syk related
protein in T cells, ZAP-70 (Chan, A.C., et al. Cell, 71:649-662 (1992)).

30   A DNA fragment encoding the 5' end of the coding sequence of *syk* was
amplified in a series of nested PCR reactions using either Syk ATG 1 or Syk ATG 2 as
the sense primer in both reactions, and ASY and ASX in the primary and secondary
reactions respectively. A standard cycling protocol was employed, with annealing of
the primers at either 60°C or 65°C. A DNA fragment of the expected size (~280 bp)

35   arose from the amplification, and after subcloning (Bluescript KS ATG 2/ASX), DNA
sequence analysis confirmed its authenticity as a partial 5' *syk* cDNA.

Because of the high error rate of Taq DNA polymerase, (Keohavong, P. and Thilly, W.G., Proc. Natl. Acad. Sci., USA, 86:9253-9257 (1980)). DNA sequence from the single 5' partial cDNA was not 100% reliable, and direct cycle sequencing of a population of PCR amplified cDNA fragments was carried out. Direct sequencing of

5   a population of PCR products effectively "neutralizes" the effect of Taq DNA polymerase infidelity (Gyllensten, U.B., and Ehrlich, H.A., Proc. Natl. Acad. Sci. USA, 85:7652-7656 (1988)). A DNA fragment encoding the first 140 amino acids of human Syk was amplified from human basophil cDNA (using the Syk ATG2/AS QALE primer pair (SEQ ID NOS: 23 and 24) isolated on a low melt agarose gel and

10   employed as template in cycle sequencing reactions using a kit (Gibco-BRL). Both primers were ^{33}P end labeled and used to obtain complementary sense and antisense DNA sequence data. This sequence data, in conjunction with that obtained from the λ phage clones, definitively determined the coding sequence for all but the first nine amino acids of the human Syk protein. Anchored PCR was then used to identify

15   these remaining nine codons and any additional 5' untranslated sequence.

Double stranded cDNA was synthesized from total human basophil RNA using a kit (Gibco-BRL) based on the method of Gubler, U., and Hoffman, B.J., Gene, 25:263-269 (1983). After the double stranded cDNA was blunted with T4 DNA polymerase (Pharmacia), an "anchor" (SEQ ID NO: 30, Anchor S and branched

20   Anchor AS, Figure 1A and 1B) was ligated onto its ends using T4 DNA ligase (Pharmacia). The anchor was a variant of that described by Roux, K.H. and Dhanarajan, P., Biotechniques: 8:48-57 (1990), in that it was designed to ligate to blunted ended rather than restriction digested DNA fragments. However, like the anchor of Roux and Dhanarajan, only the 5' end which is a substrate for ligation is

25   phosphorylated, and the other 5' end cannot hybridize to the partner strand of the anchor due to the introduction of a four base pair mismatch. This mismatch minimizes non-specific priming during PCR amplification by preventing the DNA strand covalently linked to the anchor from participating in amplification unless its complementary strand has first been specifically primed by the antisense

30   oligonucleotide in the reaction. The syk-specific antisense primers used in the amplification of the 5' end of the syk gene, ASU and ASV (SEQ ID NOS: 26 and 27, Figure 1A and 1B), were derived from the N terminal SH2 domain sequence determined by the above cycle sequencing. For detection of 5' Syk specific amplification products, Probe T (SEQ ID NO: 25), encoding sequence just 5' of ASU,

35   was also synthesized. Two consecutive PCR amplifications with nested primer pairs (1°: Uni 5'-1/ASV (SEQ ID NOS: 29 and 27 respectively), and 2° Uni 5'-2/ASU (SEQ ID NOS: 28 and 26 respectively) and the "anchored" double stranded human basophil

cDNA template were carried out under standard conditions, with an annealing temperature of 65°C. Very small DNA products (on the order of 100 bp) generated by these reactions were subcloned into Bluescript KS+ as Xho I/Cla I fragments, and sequenced. The sequence of two different inserts confirmed that the first nine amino

5   acids of human Syk are the same as those in porcine Syk. These same nine amino acids are present in the "guessmer", Syk ATG 2, used to determine the sequence of the NH$_2$ terminal SH2 domain of human Syk. Therefore, the partial 5' cDNA in Bluescript KS ATG 2/ASX possesses an intact open reading frame for the first 93 amino acids of human Syk, even though the sequence of the first nine amino acids was

10   "forced" onto the cDNA during PCR amplification.

In order to generate a cDNA spanning the entire protein coding sequence of the syk gene, it was necessary to isolate a λ gt10 phage cDNA clone from the Daudi cell library that contained the most 5' sequence of the gene and link it to the PCR generated syk 5' end clone. To find such a cDNA 44 plaques positive for

15   hybridization to the syk kinase domain (16 secondary, 28 primaries) were screened further by PCR amplification of phage supernatants with λ left and λ right primers, duplicate southern blotting of resultant products, and probing with either the human syk kinase domain probe under conditions described in (Church, G.M. and Gilbert, W., Proc. Natl. Acad. Sci. USA, 81:1991-1995 (1984), or with the Probe T from the N

20   terminal SH2 domain of human Syk (hybridization in oligo hyb mix at 60°C; washing in 2 X SSC/1% SDS at 65°C). Initially a single clone was positive for hybridization with the N terminal oligo probe but upon secondary screening with this probe, two different clones were actually pulled out. The insert that extended most 5 prime, from λ clone D5.1, was subcloned into Bluescript KS+ as an Eco RI fragment and its ends

25   sequenced. From the sequence analysis it was determined that the D5.1 cDNA extended 5' to the G$_{67}$ residue of the Syk protein. It also became apparent that the D5.1 cDNA had arisen as a consequence of random priming, rather than oligo-dT priming, since the 3' end of the clone terminated at the L$_{560}$ residue. Hence it became necessary to supplement the 3' end of the D5.1 fragment with 3' syk sequence form an

30   oligo-dT primed λ clone in addition to the 5' syk sequence from the PCR fragment.

A contiguous human syk cDNA was assembled into Bluescript SK- via a three-part ligation (Figure 2) using the internal Ban I (nucleotide 198) and Bgl II (nucleotide 1464) sites of syk and the flanking (5') Xho I/(3') Eco RI sites from partial cDNAs (Figure 2). The 5' PCR cDNA clone, Bluescript KS ATG2/AS X, was Ban I/BamHI

35   digested, and the 200 bp fragment resolved on a low melt agarose gel. Similarly, the plasmid harboring "middle" syk cDNA fragment, D5/1 was Ban I/Bgl II digested, while the 3' end of the syk gene from the λ clone T11.1 was linearized with XhoI and

Bgl II. All three fragments were mixed in a ligation and transformed into competent bacteria. Analysis of miniprep DNA identified a plasmid with the predicted restriction endonuclease sites. This plasmid, termed pBS-SK(-) hu *syk*, was subjected to complete DNA sequence analysis to confirm that it had the correct open reading

5   frame of 630 codons for human syk.

Example 2: Biospecific Interaction Analysis (BIA) In Real Time

Surface plasmon resonance (SPR), which is the basis for measurements with the BIAcore instrument (Pharmacia, Biosensor AB), is an optical phenomenon arising

10   in thin metal films under conditions of total internal reflection.

This system provides a reliable and reproducible method for the estimation of kinetic parameters. Binding is measured in real time under accurately controlled conditions, such as temperature and flow rate. Moreover, there is no need for radiolabelling of one of the components and it is not necessary to devise methods for

15   separation of bound from unbound ligand which invariably require a certain amount of time and may thus compromise the accurate measurement of kinetic parameters. One of the interacting molecules is immobilized on a dextran layer, which is in turn mounted on a gold surface. A monochromatic wedge of light is shone on this surface at a particular angle resulting in total deflection. Due to SPR, however, one

20   component of the light, termed the evanescent wave, interacts with the electrons on the gold surface, resulting in a dip in the intensity of the reflected light. The angle at which this dip is observed (resonance angle) changes with the refractive index of the medium, which in turn relates to the mass concentration very close to the gold surface. The resonance angle is measured by the instrument and converted to a plot of

25   resonance units (RU) vs. time. The higher the RU value, the higher the amount of protein at the dextran matrix. This plot is called a sensogram. The presence of compounds different from those of the running buffer results in significant "bulk effects" being measured by the BIAcore instrument, which may distort the initial phase of the association and dissociation curves. Therefore, all protein solutions are passed

30   through a desalting column equilibrated in BIAcore running buffer in order to achieve buffer exchange.

Estimation of kinetic parameters requires the repetitive injection of a range of protein concentrations over the immobilized capturing molecule. In order to be ready for a subsequent injection, the biosensor surface has to be regenerated at the end of

35   each cycle so that any non-covalently bound protein will be removed. The tyrosine-phosphorylated peptides can be used as capturing molecules, as they are not affected by relatively harsh conditions, such as injection of SDS solutions. However, the direct

covalent coupling of phosphopeptides to the dextran matrix may not be sufficient for accurate results because these small molecules may not be exposed enough to interact with the injected SH2 domains. Therefore, the biotin-avidin interaction principle can be used to place a long "spacer" between the phosphopeptides and the matrix. Avidin

5   can be covalently immobilized on the matrix and then a biotinylated phosphopeptide solution is injected. Biotinylation of the phosphopeptides can be performed using NHS biotin according to standard laboratory techniques, resulting in the covalent attachment of biotin molecules at free amino groups on the peptide. Analysis of the biotinylation mixture can be performed by reverse-phase HPLC to reveal the peaks,

10   representing varying degrees of biotinylation since both amino termini and lysine groups can be labelled. Mass analysis can also be used to identify the number of biotin molecules attached and the peaks corresponding to one biotin per peptide are used for subsequent interactions.

Association and dissociation rate constants can be calculated by repetitive

15   injections of increasing concentrations of protein over immobilized phosphopeptides, as described in the BIAcore Methods Manual. The binding of a peptide to an SH2 domain of a protein kinase is described below.

Binding of the N-SH2 peptide of PI3K p85 to pY.V.P.M.L. a PI3K ligand (SEQ ID NO: 31) was synthesized as part of a larger peptide (SEQ ID NO: 32) and

20   immobilized to the biosensor surface via the C-terminal lysine using standard EDC/NHS chemistry. To determine the binding affinity (Ka) of the N-terminal SH2 peptide of the PI3K p85 for the immobilized phosphopeptide, the on rates, off rates and steady state binding characteristics for several different concentrations of SH2 peptide (0.5, 0.67, 1.0, 2.0 and 4.0 uM) were measured from changes in the surface

25   plasmon resonance elicited by ligand/ protein interaction. The Ka for the above interaction was determined to be 160 nM.

Example 3: Mast Cell/Basophil Degranulation Assays

30   A. Intact Cell Assays

The release of β–hexosaminidase from basophils is measured as an in vitro assay of basophil activation. A cell line, RBL-2H3 (from the laboratory of Dr. Siraganian, National Institutes of Health), of rat basophil leukemia (RBL) cells is used in such an in vitro assay, according to the protocol described below (Barsumian et al.,

35   Eur. J. Immunol. 11:317-323 (1981)). The percentage of β–hexosaminidase in cells that is released into the cell medium is determined, and is a measure of the activation of the RBL-2H3 cells.

ACTIVATION OF 2BL-2H3 CELLS

MATERIALS

°RBL medium containing 2% fetal bovine serum (FBS)

5   100 ml E-MEM

         2 ml FBS

         1 ml FBS

         1.2 ml penicillin-streptomycin

filter sterilize into sterile bottles or tissue culture flasks and store at 4°C

10

°10 X PIPES buffer

         75.6 g PIPES (0.25 M)

         69.2 g NaCl (1.2 M)

         3.72 g KCl (0.05 M)

15   43 ml of 10 N NaOH (0.4 M)

         Add water to 1000 ml

filter sterilize into sterile bottles or tissue culture flasks and store at 4°C

20   °100 x calcium chloride

         1.47 g $CaCl_2 \cdot 2H_2O$ (100 mM)

         Add water to 100 ml

filter sterilize into sterile bottles or tissue culture flasks and store at 4°C

25   °2x-IgE (anti DNP-IgE mouse monoclonal antibody; Zymed) in regular RBL medium containing 15% FBS

Need 0.5 ml at 2-times an appropriate final dilution for each 16 mm well to be seeded with cells

30

°1x PIPES+glucose+calcium (prepare fresh on second day of assay)

         10 ml of 10X PIPES

         0.1 g glucose (dextrose) (5.6 Mm)

         0.1 g bovine serum albumin (BSA)

35   1 ml of 100x calcium chloride

Add water to 90 ml; pH to 7.4; milli-Q water to 100 ml

METHOD
°Remove RBL cells from flasks by trypsin-EDTA treatment (see protocol infra). Plan
to use 1×10^5 cells/16 mm well to be seeded; harvest extra cells to allow for losses

5   during centrifugation.

°Centrifuge the RBL cells and resuspend in 5 ml of regular RBL medium containing
15% FBS. Count the cells and dilute them to 2×10^5 cells/ml in the same medium.

10   °For each well to be seeded, mix 0.5 ml of cells with 0.5 ml of 2x-IgE.

°Seed 1 ml of Ige-RBL per 16 mm well.

°Incubate plate(s) overnight at 37°C/5% CO_2.

15

°Next day, dilute the DNP-HSA (dinitrophenol-human serum albumin; Calbiochem)
to an appropriate dilution in 1x PIPES+glucose+calcium; 0.5 ml will be needed for
each well.

20   °Remove the plate(s) from the incubator and aspirate the media from the wells.

°Add 2 ml of 1X PIPES+glucose+calcium to each well and aspirate again.

°Repeat the last step.

25

°Add the 0.5 ml of diluted DNP-BSA (or 1x PIPES+glucose+calcium alone as the
negative control in triplicate) to wells.

°Incubate 40 min at 37°C/5% CO_2.

30

°Meanwhile, label 12 x 75 tubes (round or conical bottom) to receive each supernatant
and the cells themselves from the triplicate negative controls; also label 12 x 75 tubes
for the beta-hexosaminidase assay.
       °   After the incubation, carefully transfer each supernatant from the 16 mm wells
35   to appropriate tubes on ice.

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°Add 250 µl of enzyme-free cell dissociation solution (Enzyme Free Cell Dissociation Solution Hank's Balanced Salts Based Formulation, Specialty Media Inc., Lavallette, NJ) to each of the triplicate negative control wells and incubate 5 min at 37°C.

5   °Transfer the 250 µl aliquots to conical bottom centrifuge tubes. Add 250 µl of PIPES+glucose+calcium to rinse each well and transfer to the appropriate tubes.

°Spin tubes 5 min at 1000 rpm and remove supernatants. Resuspend each in 500 µl of PIPES+glucose+calcium.

10

°Sonicate cell suspensions on ice (5 pulses); if no probe sonifier is available, freeze thaw cells 3x using a methanol or ethanol-dry ice bath and a 37°C water bath.

°Transfer 100 µl of each supernatant or cell lysate to a 12 x 75 tube and proceed with

15   the beta-hexosaminidase ("beta-hex") assay protocol.

Protocol for Removing RBL Cells from Flasks
°Prepare trypsin/EDTA by diluting stock 1:10 in
        HBSS--; filter sterilize.

20

°Aspirate the medium from a flask of cells.

°Add 10 ml of HBSS-- and gently rinse the flask.

25   °Aspirate the HBSS--.

°Add 3 ml of trypsin/EDTA and incubate 5 min at 37°C.

°Rap the flask several times to loosen the cells.

30

°Transfer the 3 ml to a tube.

°Rinse flask with 10 ml of the above medium and pool to the tube.

BETA-HEXOSAMINIDASE ASSAY

MATERIALS

1.   Beta-hex buffer

5   Solution 1: 0.2 M Na_2PO_4 (sodium phosphate dibasic ANHYDROUS) - 14.2 g/500 ml distilled H_2O

NOTE: If there is no anhydrous, use the sodium phosphate dibasic HEPTAHYDRATE ($7H_2O$), but use 26.7 grams.

10

Solution 2: 0.4 M citric acid monohydrate - 42.1 g/500 ml distilled H_2O

Mix approximately 70 ml of solution with approximately 20 ml solution 2 until the pH is 4.5 (use solution 1 to raise the pH and solution 2 to lower it)

15

2.   Beta-hex cocktail

Beta-hex buffer (from above): 90 ml

20   Distilled H_2O: 135 ml

p-nitrophenyl-N-acetyl-beta-D-glucosaminide (p-nitrophenyl-beta-D-2-acetamide-2-deoxy-beta-D-glucopyranoside) (Sigma N-9376): 300 mg
          (0.30 g); stored at -20°C

25

          Mix until dissolved

Aliquot in 15 ml tubes, label, date, and freeze at -20°C.

30   3.   Beta-hex STOP solution

          glycine: 15.0 g/liter
bring to pH 10.7 with N NaOH (will need to add ~30 ml of NaOH)

35

METHOD

1.   Place 100 μl of sample in each 12 x 75 polystyrene round-bottom tube.

5   2.   Add 40 μl of beta-hex cocktail to each tube.

3.   Cover with tin foil and incubate tubes 30 min at 37°C.

4.   Add 1.5 ml of beta-hex stop to each tube.

10

5.   Turn on the spectrophotometer and allow it to warm up 10 minutes.

6.   Turn on the vacuum pump attached to the spectrophotometer.

15   7.   Set the wavelength for 410 nm.

8.   Aspirate any liquid in the spectrophotometer tubing.

9.   Zero the spectrophotometer using beta-hex stop solution.

20

10. Aspirate liquid from the tubing; double check reading with more beta-hex stop.

11.   Aspirate the liquid from the tubing; read the absorbance at 410 nm (A410) of each sample within one hour, remembering to aspirate the sample from the tubing

25   between each one.

CALCULATION OF RESULTS

Determine the total beta-hex present in the RBL cells by adding the supernatant (S) and cell lysate (C) values for each of the triplicate negative controls

30   and determine the mean [S+C].

Determine the % release for each supernatant by taking the beta-hex value and dividing by (S+C) x 100.

Determine the net % release by subtracting the % release value for the control from each % release value where DNP-BSA was added.

35

B. PERMEABILIZED CELL ASSAYS

The mast cell/basophil degranulation assay can also be performed using permeabilized cells, according to the protocol described below (Cunha-Melo et al., J. Immunol. 143:2617-2625 (1989); Ali et al., J. Immunol. 143:2626-2633 (1989); Ali et al., Biochim. Biophys. Acta 1010:88-99 (1989)).

PROTOCOL FOR PERMEABILIZATION OF RBL-2H3 CELLS WITH STREPTOLYSIN O

(1) BUFFER:

Potassium glutamate	138.7 mM
Glucose	5 mM
Potassium salt of PIPES	20 mM
Magnesium Acetate	7 mM

Make 1 litre of the above buffer, do not adjust pH, sterile filter and store at 4° until use.

At the time of use, remove appropriate volume and add the following:

1 M EGTA to give final concentration of 1 mM.
ATP (Sigma #A2383) to a final concentration of 5 mM.
Add $CaCl_2$ (1 M) to a final concentration of 0.213 mM.
In the presence of 1 mM EGTA the free $[Ca^{2+}]$ is 100 nM.
Adjust pH to 7.0.
BSA (1 mg/ml) optional.
LiCl (10 to 20 nM) for phosphoinositide hydrolysis experiments.

(2) Streptolysin O
Streptolysin O reduced (Burrough's Wellcome; catalog #MR 16).

Add 4 ml H_2O to 40 I.U. of streptolysin O. (10 units/ml). Make aliquots and freeze immediately.

<u>(3) Permeabilization</u>
RBL-2H3 cells (0.3 x 10^6/well, in growth medium) are plated in 24 well tissue culture plate and incubated overnight.

5   The following day, cells are washed twice with potassium glutamate (KG) buffer (500 µl).

The cells are permeabilized by exposure to streptolysin O (0.1 to 0.3 units/ml, 200-500 µl/well) for 5 to 10 min. The concentration of streptolysin O and the time
10 required for permeabilization depends on the number of passages the cells have been cultured for and may vary with batch of streptolysin O. A useful starting point is to permeabilize cells with 0.25 I.U./ml streptolysin for 10 min.

Streptolysin O solution is prepared just before permeabilization by dilution of the
15 stock solution (10 I.U./ml) into prewarmed KG buffer.

After the cells are permeabilized, remove buffer by aspiration, add fresh buffer (without toxin) and perform experiment as desired to measure cell activation (e.g., measure phosphoinositide hydrolysis, or β–hexosaminidase or histamine release).
20 Modifications of the permeabilization procedure can be made for measuring degranulation.

Example 4: Overexpression and Purification of the C-terminal SH2 domain of human Syk
25   The sequence encoding the amino acids 163 to 265 (SEQ ID NO: 33) was amplified by the polymerase chain reaction using Taq polymerase (BMB) and oligonucleotide primers (SEQ ID NOS: 34 and 35) from the plasmid termed pBS-SK(-) hu syk. After digestion with the restriction endonucleases BamH1 (5') and EcoR1 (3'), the gel purified fragment was subcloned into the expression vector pGEX2TK using T4
30 ligase (BMB) to product the plasmid pGEX2TKCSyk(+11). The sequence of the C-terminal SH2 was determined using the chain-termination methods as described in the Sequenase (US Biochemical) protocols.
        A 2 liter culture (LB medium, 100 ug/ml ampicillin) of pGEXCSyk(+11) in transformed E. coli BL21(DE3) (Novagen) was grown at 37°C to an OD$_{595}$ of 0.432,
35 induced with 1 mM isopropyl b-D-thiogalactopyranoside (IPTG), shifted to 25 °C and harvested 12 hours later. After resuspension in phosphate buffered saline (PBS)/0.5% Triton X-100, the cells were lysed by two passes in a French Pressure Cell

at 16,000 psi. After centrifugation at 7000 x g for 20 minutes, the supernatant was purified by affinity chromatography with glutathione resin followed by elution with 20 mM glutathione in 100 mMTris pH 8.0/100 nMNaCl. This procedure yielded 25 mg/L purified fusion protein as determined by SDS-polyacrylamide gel

5 electrophoresis.

Example 5: In Vitro Transcription/Translation Assay

OBJECTIVE

10   The purpose of this assay is two fold, to determine if the following SH2 domains are functional via binding to phosphotyrosine and to develop an assay to screen and identify ligand targets (peptide or mimetics) which bind to these SH2 domains.

15 PROTOCOL

1.   The SH2 domain constructs can be subcloned into pCITE (Novagen) which has a cap-independent translation enhancer and the His-tag sequence as a C-terminal fusion allowing for the purification of the full translation product if needed. This vector has been designed for increased translation (use of 110 mM KCI during

20 translation increases the translation yield 10 fold over uncapped transcripts).

2.   The in vitro transcription can be done on each construct. The RNA is prepared using the in vitro transcription kit from Novagen. .

25 3.   The in vitro translation can be carried out using the Red Nova lysate (Novagen) using the minus leucine or minus methionine Red Nova lysate kit (for the domains that have very few to no methionines the translation can be done using H-3 leucine).

30 4.   Aliquots of the translation products can be analyzed by autoradiography on an 15-20% SDS-PAGE gel. Protein concentration can be determined using routine laboratory methods.

 5.   The full translation product can be purified using His-tag/Ni resin if there is a
35 mixture of products as determined by the autoradiograph. The protein can be eluted with imidazole.

6.   The labeled (H^3 Leu or ^{35}S Met) SH2 translation product can be incubated with the phosphotyrosine-sepharose beads (Sigma) for 30 minutes. The protein-bound beads are then washed (3 times) and an aliquot counted. The protein bound to the phosphotyrosine can either be used in competition assays or eluted with phenyl phosphate for direct binding assays as described below.

<u>ASSAYS</u>

<u>Competition for the phosphotyrosine binding</u>:

Competition experiments can also be performed using nonbiotinylated peptides or peptide mimetics to compete off the phosphotyrosine (determine IC50 for the peptide). The affinity purified (via phosphotyrosine) S-35 or H-3 labeled SH2 domains which are still bound to phosphotyrosine beads are incubated with the peptide (PDGF-R, TAM, etc.) or peptide mimetic. The sample is washed (3X), and counted. The IC$_{50}$ for different peptides (i.e., the concentration of peptide that elutes 50% of the labeled SH2 domain) is then determined.

<u>Direct Binding</u>:

The affinity purified (via phosphotyrosine) ^{35}S or H-3 labeled SH2 domains (eluted from the phosphotyrosine beads) are incubated with a peptide (e.g., TAM) and then precipitated with strepavidin-agarose beads (Pierce), washed (3X) and counted. This direct binding interaction can be quantitated by counting the ligand bound ^{35}S or H-3 labeled protein.

<u>Model System</u>

The model system for the <u>in vitro</u> transcription/ translation assay will be the lyn SH2 with the peptide KGGQY^{P}EEIPI and the N-terminal domain of PI3K with the NAcY^{P}VPMLGGK. Both protein SH2 domains can be ligated easily into the vector at the Ndel-5'/BamHI-3' sites. The peptide Y^{P}VPMLGGK (without the N-Ac) has been biotinylated and purified. Variations of this peptide as well as variations of the PDGF-R have been made available to me (J. Green) for competition studies.

These constructs will be used as the model system to develop the assay. The following nonfusion SH2 domain constructs are being subcloned into pCITE using the Ndel (5') and BamHI (3') restriction sites: lyn (U+SH3+SH2), lyn SH2 (+5 AA), PI3K NSH2.

The following N-terminal and C-terminal SH2 domains of Syk will also be cloned into the Ndel (5') and BamHI (3') using the restriction sites [NSyk M(+9) & G

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(+5) will be subcloned via the BamHI (5') and EcoR1 (3')] and tested with the phosphorylated FceRI-_ peptides. These phosphorylated peptides have been made available to me (Y. Green). These experiments can be performed as competition experiments, by competing for the phosphotyrosine-protein interaction or biotinylated

5   and done as a direct binding assay using the strepavidin beads.

NSyk (F, N-terminus)

NSyk (+9 AA & + 5 AA, M on the N-terminus and G on the C-terminus)

CSyk (+5 AA, H on the N-terminus)

CSyk (+5 AA & +12 AA, H on the N-terminus and G on the C-terminus)

10

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to be specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the

15   following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

  (i) APPLICANT: Brugge, Joan
                 Morgenstern, Jay
                 Shiue, Lily
                 Zydowsky, Lynne
                 Zoller, Mark
                 Pawson, Anthony

 (ii) TITLE OF INVENTION: Human SYK

(iii) NUMBER OF SEQUENCES: 33

 (iv) CORRESPONDENCE ADDRESS:
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      (F) ZIP: 02139

  (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

 (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER:
      (B) FILING DATE: Filed Herewith
      (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

    (A) NAME: Berstein, David L.

    (B) REGISTRATION NUMBER: 31,235

    (C) REFERENCE/DOCKET NUMBER: ARIAD 305A

(ix) TELECOMMUNICATION INFORMATION:

    (A) TELEPHONE: (617) 494-0400

    (B) TELEFAX: (617) 494-8144

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 1893 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: double

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:

        (A) NAME/KEY: CDS

        (B) LOCATION: 1..1890

    (ix) FEATURE:

        (A) NAME/KEY: N-SH2 DOMAIN

        (B) LOCATION: 28-306

    (ix) FEATURE:

        (A) NAME/KEY: C-SH2 DOMAIN

        (B) LOCATION: 487-762

    (ix) FEATURE:

        (A) NAME/KEY: KINASE DOMAIN

        (B) LOCATION: 1096-1893

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GCA GAC AGC GCG AAC CAC TTG CCC TTC TTT TTC GGC AAC ATC ACC        48
Met Ala Asp Ser Ala Asn His Leu Pro Phe Phe Phe Gly Asn Ile Thr
 1               5                  10                  15


CGG GAG GAG GCA GAA GAT TAC CTG GTC CAG GGG GGC ATG AGT GAT GGG        96
Arg Glu Glu Ala Glu Asp Tyr Leu Val Gln Gly Gly Met Ser Asp Gly
            20                  25                  30


CTT TAT TTG CTG CGC CAG AGC CGC AAC TAC CTG GGT GGC TTC GCC CTG       144
Leu Tyr Leu Leu Arg Gln Ser Arg Asn Tyr Leu Gly Gly Phe Ala Leu
            35                  40                  45


TCC GTG GCC CAC GGG AGG AAG GCA CAC CAC TAC ACC ATC GAG CGG GAG       192
Ser Val Ala His Gly Arg Lys Ala His His Tyr Thr Ile Glu Arg Glu
        50                  55                  60


CTG AAT GGC ACC TAC GCC ATC GCC GGT GGC AGG ACC CAT GCC AGC CCC       240
Leu Asn Gly Thr Tyr Ala Ile Ala Gly Gly Arg Thr His Ala Ser Pro
65                  70                  75                  80


GCC GAC CTC TGC CAC TAC CAC TCC CAG GAG TCT GAT GGC CTG GTC TGC       288
Ala Asp Leu Cys His Tyr His Ser Gln Glu Ser Asp Gly Leu Val Cys
                85                  90 .                95


CTC CTC AAG AAG CCC TTC AAC CGG CCC AAG GGG TGC AGC CCG AAG ACT       336
Leu Leu Lys Lys Pro Phe Asn Arg Pro Lys Gly Cys Ser Pro Lys Thr
            100                 105                 110


GGG CCC TTT GAG GAT TTG AAG GAA AAC CTC ATC AGG GAA TAT GTG AAG       384
Gly Pro Phe Glu Asp Leu Lys Glu Asn Leu Ile Arg Glu Tyr Val Lys
            115                 120                 125


CAG ACA TGG AAC CTG CAG GGT CAG GCT CTG GAG CAG GCC ATC ATC AGT       432
Gln Thr Trp Asn Leu Gln Gly Gln Ala Leu Glu Gln Ala Ile Ile Ser
            130                 135                 140
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CAG AAG CCT CAG CTG GAG AAG CTG ATC GCT ACC ACA GCC CAT GAA AAA          480
Gln Lys Pro Gln Leu Glu Lys Leu Ile Ala Thr Thr Ala His Glu Lys
145             150             155             160


ATG CCT TGG TTC CAT GGA AAA ATC TCT CGG GAA GAA TCT GAG CAA ATT          528
Met Pro Trp Phe His Gly Lys Ile Ser Arg Glu Glu Ser Glu Gln Ile
            165             170             175


GTC CTG ATA GGA TCA AAG ACA AAT GGA AAG TTC CTG ATC CGA GCC AGA          576
Val Leu Ile Gly Ser Lys Thr Asn Gly Lys Phe Leu Ile Arg Ala Arg
            180             185             190


GAC AAC AAC GGC TCC TAC GCC CTG TGC CTG CTG CAC GAA GGG AAG GTG          624
Asp Asn Asn Gly Ser Tyr Ala Leu Cys Leu Leu His Glu Gly Lys Val
            195             200             205


CTG CAC TAT CGC ATC GAC AAA GAC AAG ACA GGG AAG CTC TCC ATC CCC          672
Leu His Tyr Arg Ile Asp Lys Asp Lys Thr Gly Lys Leu Ser Ile Pro
            210             215             220


GAG GGA AAG AAG TTC GAC ACG CTC TGG CAG CTA GTC GAG CAT TAT TCT          720
Glu Gly Lys Lys Phe Asp Thr Leu Trp Gln Leu Val Glu His Tyr Ser
225             230             235             240


TAT AAA GCA GAT GGT TTG TTA AGA GTT CTT ACT GTC CCA TGT CAA AAA          768
Tyr Lys Ala Asp Gly Leu Leu Arg Val Leu Thr Val Pro Cys Gln Lys
            245             250             255


ATC GGC ACA CAG GGA AAT GTT AAT TTT GGA GGC CGT CCA CAA CTT CCA          816
Ile Gly Thr Gln Gly Asn Val Asn Phe Gly Gly Arg Pro Gln Leu Pro
            260             265             270


GGT TCC CAT CCT GCG ACT TGG TCA GCG GGT GGA ATA ATC TCA AGA ATC          864
Gly Ser His Pro Ala Thr Trp Ser Ala Gly Gly Ile Ile Ser Arg Ile
            275             280             285
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AAA TCA TAC TCC TTC CCA AAG CCT GGC CAC AGA AAG TCC TCC CCT GCC        912
Lys Ser Tyr Ser Phe Pro Lys Pro Gly His Arg Lys Ser Ser Pro Ala
    290                 295                 300


CAA GGG AAC CGG CAA GAG AGT ACT GTG TCA TTC AAT CCG TAT GAG CCA        960
Gln Gly Asn Arg Gln Glu Ser Thr Val Ser Phe Asn Pro Tyr Glu Pro
305                 310                 315                 320


GAA CTT GCA CCC TGG GCT GCA GAC AAA GGC CCC CAG AGA GAA GCC CTA       1008
Glu Leu Ala Pro Trp Ala Ala Asp Lys Gly Pro Gln Arg Glu Ala Leu
                325                 330                 335


CCC ATG GAC ACA GAG GTG TAC GAG AGC CCC TAC GCG GAC CYC GAG GAG       1056
Pro Met Asp Thr Glu Val Tyr Glu Ser Pro Tyr Ala Asp Pro Glu Glu
                340                 345                 350


ATC AGG CCC AAG GAG GTT TAC CTG GAC CGA AAG CTG CTG ACG CTG GAA       1104
Ile Arg Pro Lys Glu Val Tyr Leu Asp Arg Lys Leu Leu Thr Leu Glu
                355                 360                 365


GAC AAA GAA CTG GGC TCT GGT AAT TTT GGA ACT GTG AAA AAG GGC TAC       1152
Asp Lys Glu Leu Gly Ser Gly Asn Phe Gly Thr Val Lys Lys Gly Tyr
    370                 375                 380


TAC CAA ATG AAA AAA GTT GTG AAA ACC GTG GCT GTG AAA ATA CTG AAA       1200
Tyr Gln Met Lys Lys Val Val Lys Thr Val Ala Val Lys Ile Leu Lys
385                 390                 395                 400


AAC GAG GCC AAT GAC CCC GCT CTT AAA GAT GAG TTA TTA GCA GAA GCA       1248
Asn Glu Ala Asn Asp Pro Ala Leu Lys Asp Glu Leu Leu Ala Glu Ala
                405                 410                 415


AAT GTC ATG CAG CAG CTG GAC AAC CCG TAC ATC GTG CGR ATG ATC GGG       1296
Asn Val Met Gln Gln Leu Asp Asn Pro Tyr Ile Val Arg Met Ile Gly
                420                 425                 430
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ATA TGC GAG GCC GAG TCC TGG ATG CTR GTT ATG GAG ATG GCA GAA CTT          1344
Ile Cys Glu Ala Glu Ser Trp Met Leu Val Met Glu Met Ala Glu Leu
        435                 440                 445


GGT CCC CTC AAT AAG TAT TTG CAG CAG AAC AGA CAT GTC AAG GAT AAG          1392
Gly Pro Leu Asn Lys Tyr Leu Gln Gln Asn Arg His Val Lys Asp Lys
        450                 455                 460


AAC ATC ATA GAA CTG GTT CAT CAG GTT TCC ATG GGC ATG AAG TAC TTG          1440
Asn Ile Ile Glu Leu Val His Gln Val Ser Met Gly Met Lys Tyr Leu
465                 470                 475                 480


GAG GAG AGC AAT TTT GTG CAC AGA GAT CTG GCT GCA AGA AAT GTG TTG          1488
Glu Glu Ser Asn Phe Val His Arg Asp Leu Ala Ala Arg Asn Val Leu
                485                 490                 495


CTA GTT ACC CAA CAT TAC GCC AAG ATC AGT GAT TTC GGA CTT TCC AAA          1536
Leu Val Thr Gln His Tyr Ala Lys Ile Ser Asp Phe Gly Leu Ser Lys
                500                 505                 510


GCA CTG CGT GCT GAT GAA AAC TAC TAC AAG GCC CAG ACC CAT GGA AAG          1584
Ala Leu Arg Ala Asp Glu Asn Tyr Tyr Lys Ala Gln Thr His Gly Lys
        515                 520                 525


TGG CCT GTC AAG TGG TAC GCT CCG GAA TGC ATC AAC TAC TAC AAG TTC          1632
Trp Pro Val Lys Trp Tyr Ala Pro Glu Cys Ile Asn Tyr Tyr Lys Phe
        530                 535                 540


TCC AGC AAA AGC GAT GTC TGG AGC TTT GGA GTG TTG ATG TGG GAA GCA          1680
Ser Ser Lys Ser Asp Val Trp Ser Phe Gly Val Leu Met Trp Glu Ala
545                 550                 555                 560


TTC TCC TAT GGG CAG AAG CCA TAT CGA GGG ATG AAA GGA AGT GAA GTC          1728
Phe Ser Tyr Gly Gln Lys Pro Tyr Arg Gly Met Lys Gly Ser Glu Val
                565                 570                 575
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ACC GCT ATG TTA GAG AAA GGA GAG CGG ATG GGG TGC CCT GCA GGG TGT      1776
Thr Ala Met Leu Glu Lys Gly Glu Arg Met Gly Cys Pro Ala Gly Cys
            580                 585                 590


CCA AGA GAG ATG TAC GAT CTC ATG AAT CTG TGC TGG ACA TAC GAT GTG      1824
Pro Arg Glu Met Tyr Asp Leu Met Asn Leu Cys Trp Thr Tyr Asp Val
            595                 600                 605


GAA AAC AGG CCC GGA TTC GCA GCA GTG GAA CTG CGG CTG CGC AAT TAC      1872
Glu Asn Arg Pro Gly Phe Ala Ala Val Glu Leu Arg Leu Arg Asn Tyr
            610                 615                 620


TAC TAT GAC GTG GTG AAC TAA                                          1893
Tyr Tyr Asp Val Val Asn
625                 630
```

(2) INFORMATION FOR SEQ ID NO:2:

      (i) SEQUENCE CHARACTERISTICS:

          (A) LENGTH: 630 amino acids

          (B) TYPE: amino acid

          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:


```
Met Ala Asp Ser Ala Asn His Leu Pro Phe Phe Phe Gly Asn Ile Thr
1                 5                 10                  15


Arg Glu Glu Ala Glu Asp Tyr Leu Val Gln Gly Gly Met Ser Asp Gly
            20                  25                  30


Leu Tyr Leu Leu Arg Gln Ser Arg Asn Tyr Leu Gly Gly Phe Ala Leu
            35                  40                  45
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Ser Val Ala His Gly Arg Lys Ala His His Tyr Thr Ile Glu Arg Glu
    50              55              60

Leu Asn Gly Thr Tyr Ala Ile Ala Gly Gly Arg Thr His Ala Ser Pro
65              70              75              80

Ala Asp Leu Cys His Tyr His Ser Gln Glu Ser Asp Gly Leu Val Cys
                85              90              95

Leu Leu Lys Lys Pro Phe Asn Arg Pro Lys Gly Cys Ser Pro Lys Thr
            100             105             110

Gly Pro Phe Glu Asp Leu Lys Glu Asn Leu Ile Arg Glu Tyr Val Lys
        115             120             125

Gln Thr Trp Asn Leu Gln Gly Gln Ala Leu Glu Gln Ala Ile Ile Ser
    130             135             140

Gln Lys Pro Gln Leu Glu Lys Leu Ile Ala Thr Thr Ala His Glu Lys
145             150             155             160

Met Pro Trp Phe His Gly Lys Ile Ser Arg Glu Glu Ser Glu Gln Ile
            165             170             175

Val Leu Ile Gly Ser Lys Thr Asn Gly Lys Phe Leu Ile Arg Ala Arg
            180             185             190

Asp Asn Asn Gly Ser Tyr Ala Leu Cys Leu Leu His Glu Gly Lys Val
            195             200             205

Leu His Tyr Arg Ile Asp Lys Asp Lys Thr Gly Lys Leu Ser Ile Pro
    210             215             220

Glu Gly Lys Lys Phe Asp Thr Leu Trp Gln Leu Val Glu His Tyr Ser
225             230             235             240
```

Tyr Lys Ala Asp Gly Leu Leu Arg Val Leu Thr Val Pro Cys Gln Lys
        245   250   255

Ile Gly Thr Gln Gly Asn Val Asn Phe Gly Gly Arg Pro Gln Leu Pro
        260   265   270

Gly Ser His Pro Ala Thr Trp Ser Ala Gly Gly Ile Ile Ser Arg Ile
        275   280   285

Lys Ser Tyr Ser Phe Pro Lys Pro Gly His Arg Lys Ser Ser Pro Ala
        290   295   300

Gln Gly Asn Arg Gln Glu Ser Thr Val Ser Phe Asn Pro Tyr Glu Pro
305   310   315   320

Glu Leu Ala Pro Trp Ala Ala Asp Lys Gly Pro Gln Arg Glu Ala Leu
                325   330   335

Pro Met Asp Thr Glu Val Tyr Glu Ser Pro Tyr Ala Asp Pro Glu Glu
                340   345   350

Ile Arg Pro Lys Glu Val Tyr Leu Asp Arg Lys Leu Leu Thr Leu Glu
        355   360   365

Asp Lys Glu Leu Gly Ser Gly Asn Phe Gly Thr Val Lys Lys Gly Tyr
370   375   380

Tyr Gln Met Lys Lys Val Val Lys Thr Val Ala Val Lys Ile Leu Lys
385   390   395   400

Asn Glu Ala Asn Asp Pro Ala Leu Lys Asp Glu Leu Leu Ala Glu Ala
                405   410   415

Asn Val Met Gln Gln Leu Asp Asn Pro Tyr Ile Val Arg Met Ile Gly
                420   425   430

-41-

```
Ile Cys Glu Ala Glu Ser Trp Met Leu Val Met Glu Met Ala Glu Leu
        435             440             445

Gly Pro Leu Asn Lys Tyr Leu Gln Gln Asn Arg His Val Lys Asp Lys
        450             455             460

Asn Ile Ile Glu Leu Val His Gln Val Ser Met Gly Met Lys Tyr Leu
465             470             475             480

Glu Glu Ser Asn Phe Val His Arg Asp Leu Ala Ala Arg Asn Val Leu
            485             490             495

Leu Val Thr Gln His Tyr Ala Lys Ile Ser Asp Phe Gly Leu Ser Lys
        500             505             510

Ala Leu Arg Ala Asp Glu Asn Tyr Tyr Lys Ala Gln Thr His Gly Lys
        515             520             525

Trp Pro Val Lys Trp Tyr Ala Pro Glu Cys Ile Asn Tyr Tyr Lys Phe
        530             535             540

Ser Ser Lys Ser Asp Val Trp Ser Phe Gly Val Leu Met Trp Glu Ala
545             550             555             560

Phe Ser Tyr Gly Gln Lys Pro Tyr Arg Gly Met Lys Gly Ser Glu Val
            565             570             575

Thr Ala Met Leu Glu Lys Gly Glu Arg Met Gly Cys Pro Ala Gly Cys
        580             585             590

Pro Arg Glu Met Tyr Asp Leu Met Asn Leu Cys Trp Thr Tyr Asp Val
        595             600             605
```

Glu Asn Arg Pro Gly Phe Ala Ala Val Glu Leu Arg Leu Arg Asn Tyr
  610   615   620

Tyr Tyr Asp Val Val Asn
625   630

(2) INFORMATION FOR SEQ ID NO:3:

  (i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 28 base pairs

    (B) TYPE: nucleic acid

    (C) STRANDEDNESS: single

    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCTCGAGTT YTTYTTYGGN CARATTAC   28

(2) INFORMATION FOR SEQ ID NO:4:

  (i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 28 base pairs

    (B) TYPE: nucleic acid

    (C) STRANDEDNESS: single

    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCTCGAGTT YTTYTTYGGN CARATAAC   28

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 28 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCTCGAGTT YTTYTTYGGN CARATCAC   28

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 32 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCATCGATC CRTGRAACCA NGGCATYTTT TC   32

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 32 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCATCGATC CRTGRAACCA NGGCATYTTC TC   32

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 31 base pairs

       (B) TYPE: nucleic acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCTCGAGTG GATGCTDGTN ATGGAAATGG C   31

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 31 base pairs

       (B) TYPE: nucleic acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCTCGAGTG GATGCTDGTN ATGGAGATGG C   31

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 34 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCATCGATA RTCHACHACR TCRTARTART AGTT   34

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 33 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCATCGATA RTCHACHACR TCRTADARTA ATT   33

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 21 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGTATTTGCA GCAGAACCAG A   21

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 32 base pairs

       (B) TYPE: nucleic acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCATCGATA TGATGTTCTT ATCCTTGACA TG   32

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 24 base pairs

       (B) TYPE: nucleic acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CATGGAAACC TGATGAACCA GTTC   24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 25 base pairs

    (B) TYPE: nucleic acid

    (C) STRANDEDNESS: single

    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GACTGCTGGG TAGTCCCCAC CTTTT   25

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 25 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAGCAAGTTC AGCCTGGTTA AGTCC   25

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 26 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGGGTAAAAT AACAGAGGTG GCTTAT   26

(2) INFORMATION FOR SEQ ID NO:18:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 25 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAGTATTTCT TCCAGGGTAA AAAGC   25

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 21 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAGCCCCGCC GACCTCTGCC A   21

(2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 33 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCCATCGATC CATCAGACTC CTGGGAGTGG TAG   33

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 24 base pairs

      (B) TYPE: nucleic acid

      (C) STRANDEDNESS: single

      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGCTTCTTGA GGAGGCAGAC CAGG   24

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 34 base pairs

      (B) TYPE: nucleic acid

      (C) STRANDEDNESS: single

      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGCTCGAGAT GGCAGACAGY GCSAAYCACC TGCC   34

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 34 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGCTCGAGAT GGCAGACAGY GCSAAYCACT TGCC   34

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 24 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATGATGGCCT GCTCCAGAGC CTGA   24

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 23 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATCACCCGGG AGGAGGCWGA AGA   23

(2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCCATCGATG CCACCCAGGT AGTTGCGGCT CTG   33

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTCCTGGGAG TGGTAGTGGC AGAG   24

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CAATTAACCC CTCGAGAATT CCCAA   25

(2) INFORMATION FOR SEQ ID NO:29:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCAATGAACG ATCATGTAGC AATGC   25

(2) INFORMATION FOR SEQ ID NO:30:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 50 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TCAATGAACG ATCATGTAGC AATGCCAATT AACCCCTCGA GAATTCCCAA   50

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 103 amino acids

    (B) TYPE: amino acid

    (C) STRANDEDNESS: single

    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Trp Phe His Gly Lys Ile Ser Arg Glu Glu Ser Glu Gln Ile Val Leu
1   5   10   15

Ile Gly Ser Lys Thr Asn Gly Lys Phe Leu Ile Arg Ala Arg Asp Asn
                20   25   30

Asn Gly Ser Tyr Ala Leu Cys Leu Leu His Glu Gly Lys Val Leu His
                35   40   45

Tyr Arg Ile Asp Lys Asp Lys Thr Gly Lys Ile Ser Ile Pro Glu Gly
        50   55   60

Lys Lys Phe Asp Thr Leu Trp Gln Leu Val Glu His Tyr Ser Tyr Lys
65   70   75   80

Ala Asp Gly Leu Leu Arg Val Leu Thr Val Pro Cys Gln Lys Ile Gly
                85   90   95

Thr Gln Gly Asn Val Asn Phe
                100

(2) INFORMATION FOR SEQ ID NO:32: .

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 42 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: Synthetic DNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGCGCGAAGC TTGGATCCTG GTTCCATGGA AAAATCTCTC GG   42

(2) INFORMATION FOR SEQ ID NO:33:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 35 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: Synthetic DNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCGAATTCTC AAAAATTAAC ATTTCCCTGT GTGCC   35

CLAIMS

The invention claimed is:

1.   An isolated DNA molecule comprising a DNA sequence encoding part or all of the amino acid sequence spanning positions Met-1 through Gly-180 of SEQ ID NO:2, or allelic variants thereof.

2.   An isolated DNA molecule comprising part or all of the nucleotide sequence spanning positions A-1 through A-540 of SEQ ID NO:1, or allelic variants thereof.

3.   An isolated DNA molecule comprising the nucleotide sequence spanning positions A-1 through C-1890 of SEQ ID NO:1, or allelic variants thereof.

4.   A recombinant DNA construct comprising a DNA molecule of claims 1, 2 or 3, and heterologous DNA.

5.   A genetically engineered host cell containing a recombinant DNA construct of claim 4.

```
ggctcgag tt(tc) tt(tc) tt(tc) gg(gatc) ca(ag) att ac        syk S1A
ggctcgag tt(tc) tt(tc) tt(tc) gg(gatc) ca(ag) ata ac        syk S1B
ggctcgag tt(tc) tt(tc) tt(tc) gg(gatc) ca(ag) atc ac        syk S1C
 Xho I    F      F      F      G        Q      I   T

cccatcgat cc (ag)tg (ag)aa cca (gatc)gg cat (tc)tt ttc      syk AS2A
cccatcgat cc (ag)tg (ag)aa cca (gatc)gg cat (tc)tt ctc      syk AS2B
 Cla I     G  H       F      W    P        M    K      E

ggctcgag tgg atg ct(gat) gt(gatc) atg gaa atg gc            syk S3A
ggctcgag tgg atg ct(gat) gt(gatc) atg gag atg gc            syk S3B
 Xho I    W   M   L       V        M   E   M   A

cccatcgat a (ag)tc (atc)ac (atc)ac (ag)tc (ag)ta (ag)ta (ag)ta gtt   syk AS4A
cccatcgat a (ag)tc (atc)ac (atc)ac (ag)tc (ag)ta (ag)ta (agt)a att   syk AS4B
 Cla I      N      V        V        D       Y       Y       Y   N

agt att tgc agc aga acc aga                                  syk Probe Z
```

Figure 1A / 2

syk ASA

cccatcgat at gat gtt ctt atc ctt gac atg
ClaI L I N K D K V H

syk ASB

cat gga aac ctg atg aac cag ttc
M S V Q H V L E

λ for 1　gactgctggtagtcccacctttt
λ for 2　gagcaagttcagcctggttaagtcc
λ rev 1　ggggtaaataacagaggtggcttat
λ rev 2　gagtatttcttccagggtaaaaagc

syk Probe W

cag ccc cgc cga cct ctg cca
S P A D L C H

syk ASX

cccatcgat cca tca gac tcc tgg gag tgg tag
ClaI G D S E Q S H Y

syk ASY

ggc ttc ttg agg agg cag acc agg
P K K L L C V L

syk ATG 1

ggctcgag atg gca gac ag(ct) gc(gc) aa(ct) cac ctg cc
M A D S A N H L P

syk ATG 2

ggctcgag atg gca gac ag(ct) gc(gc) aa(ct) cac ttg cc
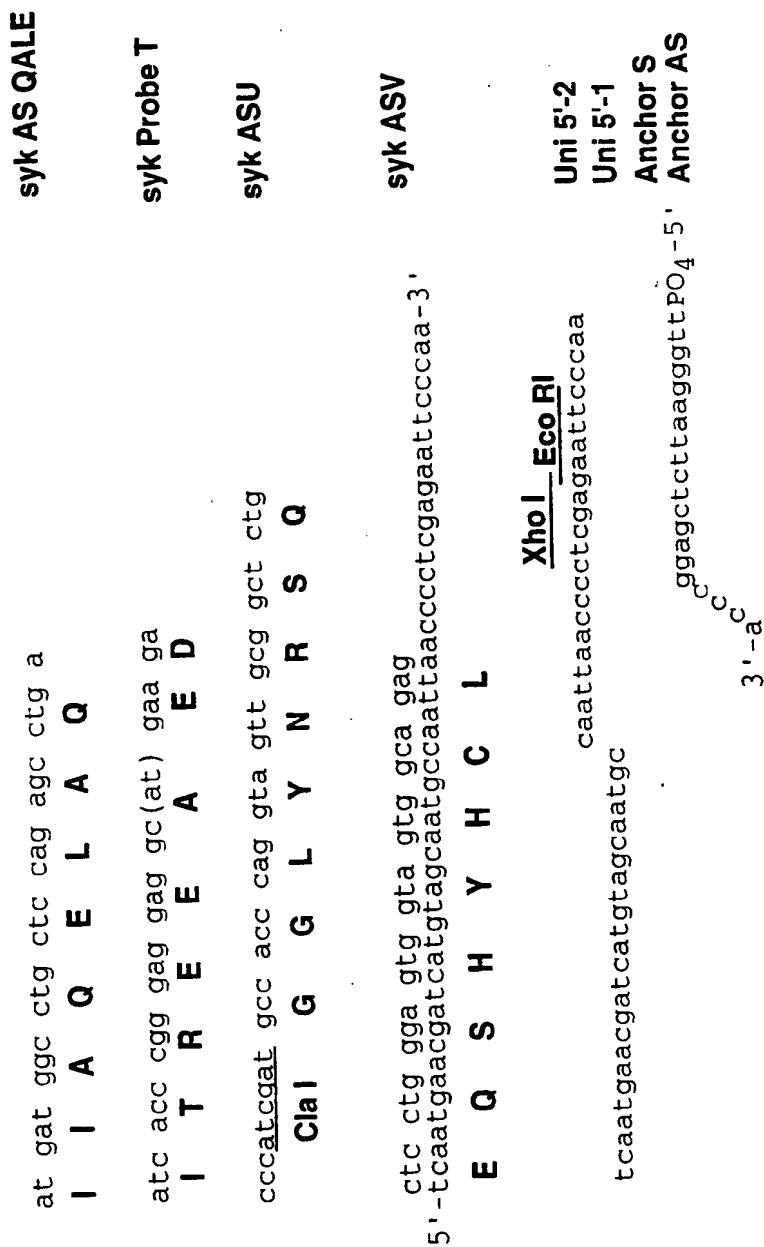XhoI M A D S A N H L P

Figure 1B / 2

SUBSTITUTE SHEET (RULE 26)

at gat ggc ctg ctc cag agc ctg a   **syk AS QALE**
 I   I   A   Q   E   L   A   Q

atc acc cgg gag gag gc(at) gaa ga   **syk Probe T**
 I   T   R   E   E   A   E   D

<u>cccatcgat</u> gcc acc cag gta gtt gcg gct ctg   **syk ASU**
ClaI   G   G   L   Y   N   R   S   Q

                ctc ctg gga gtg gta gtg gca gag   **syk ASV**
5'-tcaatgaacgatcatgtagcaattgccaattaacccctcgagaattcccaa-3'
                 E   Q   S   H   Y   H   C   L

                         <u>XhoI</u> <u>EcoRI</u>
                  caattaaccccctcgagaattcccaa   **Uni 5'-2**
                                                          Uni 5'-1

                                                          Anchor S
tcaatgaacgatcatgtagcaatgc   **Anchor AS**

                                    c
                                   c
                      3'-a c ggagctcttaagggttPO$_4$-5'

Figure 1C / 2

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Figure 2 / 2

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 1/11, 1/15, 5/10, 15/12
US CL :536/23.5; 435/240.1, 252.3, 255, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/240.1, 252.3, 255, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS
search terms: syk, SH2, kinase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 71, issued 13 November 1992, Chan et al., "ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TRC zeta chain", pages 649-662, see whole publication, especially Figure 5, particularly Figure 5 on page 654.	1-5
Y	Science, Volume 239, issued 11 March 1988, Lee et al., "Generation of cDNA probes directed by amino acid sequence: cloning of urate oxidase", pages 1288-1291, see whole publication, especially pages the abstract and pages 1289 and 1291.	1-5

| X | Further documents are listed in the continuation of Box C. | ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 AUGUST 1994	10 AUG 1994

| Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Telephone No. (703) 308-0196 |

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The Journal of Biological Chemistry, Volum 267, Number 35, issued 15 December 1992, Samelson et al., "Tyrosine kinases and tyrosine based activation motifs", pages 24913-24916, see whole publication.	1-5

```
ggctcgag tt(tc) tt(tc) tt(tc) gg(gatc) ca(ag) att ac                          syk S1A
ggctcgag tt(tc) tt(tc) tt(tc) gg(gatc) ca(ag) ata ac                          syk S1B
ggctcgag tt(tc) tt(tc) tt(tc) gg(gatc) ca(ag) atc ac                          syk S1C
 Xho I     F      F      F      G       Q      I   T

cccatcgat cc (ag)tg (ag)aa cca (gatc)gg cat (tc)tt ttc                         syk AS2A
cccatcgat cc (ag)tg (ag)aa cca (gatc)gg cat (tc)tt ctc                         syk AS2B
 Cla I      G      H      F       W       P       M   K   E

ggctcgag tgg atg ct(gat) gt(gatc) atg gaa atg gc                              syk S3A
ggctcgag tgg atg ct(gat) gt(gatc) atg gag atg gc                              syk S3B
 Xho I     W   M   L       V        M   E   M   A

cccatcgat a (ag)tc (atc)ac (atc)ac (ag)tc (ag)tc (ag)ta (ag)ta gtt            syk AS4A
cccatcgat a (ag)tc (atc)ac (atc)ac (ag)tc (ag)ta (agt)a (ag)a att             syk AS4B
 Cla I      N       V       Y       D       Y      Y      N

agt att tgc agc aga acc aga                                                   syk Probe Z
```

Figure 1A / 2

```
cccatcgat at gat gtt ctt atc ctt gac atg          syk ASA
ClaI      L   I   N   K   D   K   V   H

cat gga aac ctg atg aac cag ttc                   syk ASB
    M   S   V   Q   H   V   L   E

gactgctggtagtcccacttt                             λ for 1
gagcaagttcagcctggtaagtcc                          λ for 2
ggggtaaaataacagagggttat                           λ rev 1
gagtattcttccagggtaaaagc                           λ rev 2

cag ccc cgc cga cct ctg cca                       syk Probe W
    S   P   A   D   L   C   H

cccatcgat cca tca gac tcc tgg gag tgg tag         syk ASX
ClaI      G   D   S   E   Q   S   H   Y

ggc ttc ttg agg agg cag acc agg                   syk ASY
    P   K   K   L   L   C   V   L

ggctcgag atg gca gac ag(ct) gc(gc) aa(ct) cac ctg cc   syk ATG 1

ggctcgag atg gca gac ag(ct) gc(gc) aa(ct) cac ttg cc   syk ATG 2
XhoI     M   A   D   S      A      N      H   L   P
```

Figure 1B / 2

SUBSTITUTE SHEET (RULE 26)

BNSDOCID: <WO___9425565A1TI_>

at gat ggc ctg ctc cag agc ctg a   syk AS QALE
I   I   A   Q   E   L   A   Q

atc acc cgg gag gag gc(at) gaa ga   syk Probe T
I   T   R   E   E   A   E   D

cccatcgat gcc acc cag gta gtt gcg gct ctg   syk ASU
ClaI   G   G   L   Y   N   R   S   Q

ctc ctg gga gtg gta gtg gca gag   syk ASV
5'-tcaatgaacgatcatgtagcaatgccaattaacccctcgagaattcccaa-3'
E   Q   S   H   Y   H   C   L

__XhoI__ __Eco RI__
caattaaccccctcgagaattccccaa   Uni 5'-2
     Uni 5'-1

tcaatgaacgatcatgtagcaatgc   Anchor S
     Anchor AS
    ggagctcttaagggttPO₄-5'
3'-a

Figure 1C / 2

Figure 2 / 2

BNSDOCID: <WO___9425565A1TI_>